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Hormonal Correlates of Coloration and Sexual Change in the Hermaphroditic Grouper, *Epinephelus adscensionis*

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Hormonal Correlates of Coloration and Sexual Change in the Hermaphroditic Grouper, *Epinephelus adscensionis*

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Dedication

To Kristen Davis who supported me throughout this entire process and is now an expert in “grouperology”

&

To the late Joe Kuban, a mentor who showed me how fun science can be.

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I thank the rock hind *Epinephelus adscensionis* for being amazing models to test my hypothesis. I do not take lightly the samples that I have collected from them. I thank the members of my dissertation committee for their time and generous cooperation in this research: my co-supervisors Joan Holt and Izhar Khan for spending innumerable hours helping me shape my observations and experimental results regarding the rock hind; Hans Hofmann, for the generous use of his lab and teaching me the art of immunocytochemistry; John Godwin for his insights on the AVT system and advice on sex changing fish models; and Peter Thomas for his help with experimental design and the generous use of his laboratory. I also thank Md. Saydur Rahman for his constant encouragement and guidance with molecular experiments. I thank Kristen Davis for her help with just about every aspect of this dissertation. I also thank everyone at FAML for their encouragement and assistance, particularly Venus Mills, Jeff Kaiser, Cindy Faulk, Ken Webb, and J. Shaik Mohamed. I also thank my numerous dive buddies for the long hours underwater observing and collecting rock hind, especially John Williams, David Moore, Luke Dotson and Jay Rooker. I thank Erich Schlegel and Robert Patzner for providing photographs of rock hind that I used in presentations and this study. Finally I thank my parents for their undying support in all of my adventures.

Hormonal Correlates of Coloration and Sexual Change in the Hermaphroditic Grouper, *Epinephelus adscensionis*

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Hermaphroditism, associated with territoriality and dominance behavior, is common in the marine environment. Male sex-specific coloration patterns and behavior are particularly evident in species where males are territorial and guard harems of females such as wrasses and groupers. Protogynous hermaphrodites that change sex from female to male are good models to study sexual behavior and related changes in the brain due to their abilities to reorganize their sexual phenotype as adults. Two hormones produced in the brain and implicated in the process of sex-specific behavior and reproductive development are arginine vasotocin (AVT) and gonadotropin releasing hormone (GnRH). While a wealth of data exists regarding these hormone systems separately, little is known about linkage between these two systems. Especially there is no data tracking these two systems together in any protogynous fish. This study was conducted to test the hypothesis that coordinated interactions between AVT and GnRH facilitate the process of behavioral and gonadal sex change in the rock hind Epinephelus adscensionis. Four topics were addressed to investigate the relationship between behavior and reproduction: i) rock hind

sex change, sexual characteristics and conditions causing sex change to occur in captivity were detailed as a basis for examining the AVT system and GnRH during this process, ii) the distribution of a vasotocin V1a type receptor identified in rock hind brain was examined for the first time in a fish species using a custom designed antibody then the receptor protein was co-localized with GnRH producing cells within the brain to confirm that a pathway exists for AVT action on GnRH, iii) levels of AVT, AVT receptors, and GnRH messenger RNA (mRNA) were compared between male and female rock hind phenotypes, and iv) female rock hind at early stages of sex change were compared for brain mRNA expression of AVT, AVT receptors, and GnRH to determine the order of hormonal change during the process of sexual inversion in this species. This study provides a better understanding of the relationship between sex-specific behavior and reproductive development via AVT and GnRH systems that are conserved in all vertebrates.

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Chapter 1: General Introduction

Sexual behavior and development are controlled by hormones that signal changes in response to stimuli from the internal and external environment. Small peptide hormones, due to their production in specific brain areas and rapid action via receptors on or within the membrane surface of cells are good candidates for mediating behavioral change. Peptide hormones also circulate readily in the bloodstream and are transmitted through nervous tissue causing rapid actions in cells via binding of receptors on the surface of cells (Strader et al., 1994).

The neuropeptide hormones arginine vasotocin (AVT) and the mammalian homologue arginine vasopressin (AVP) have been demonstrated in various vertebrate phyla to be associated with numerous physiological processes such as osmoregulation, circulatory function and the stress response. Behavioral functions especially related to sex specific behaviors of aggression, territoriality, and sonic communication and mate recognition have been correlated with the production of AVT/AVP (see reviews by: Bass and Grober, 2001; Foran and Bass, 1999b). In fish, the main production site for this hormone is the preoptic and anterior hypothalamus (POAH), a brain region for synthesis of many of the hormones that regulate reproduction and behavior in all vertebrates (Foran and Bass, 1999b). In birds and amphibians, other regions of the brain are also known to produce this hormone (Boyd, 1997; Jurkevich et al., 1996). In Salmon and suckerfish (Morley et al., 1990), two AVT forms have been identified while only one form has been reported in most other fish species (Balment et al., 2006a).

Gonadotropin releasing hormone (GnRH) is a decapeptide hormone that occurs in all vertebrates (Dubois et al., 2002). The GnRH by definition stimulates release of the

two gonadotropins from the pituitary: follicle stimulating hormone and luteinizing hormone, which in turn regulate gonadal development and growth (Powell et al., 1994). Two or more GnRH forms are present in all vertebrates: the releasing form originating in the preoptic area (GnRH-I), GnRH-II in the midbrain of all vertebrates, and in some cases, a third form known as GnRH-III. Behavioral changes due to different forms of GnRH have been demonstrated in some fish species but not in others. Changes in abundance and size of GnRH neurons and/or GnRH mRNA or peptide levels have been shown to vary between the sexes and reproductive state in both gonochoristic and hermaphroditic fishes (Maruska et al., 2007a; Mohamed et al., 2005; Parhar et al., 2001; Senthilkumaran et al., 1999a; White et al., 2002).

The GnRH is linked to sexual state in many fish species. In anemone fish the number of preoptic area GnRH cells is significantly higher in male phase rather than juvenile or female stage indicating a role in gonadal and or behavioral sex (Elofsson et al., 1997). In the gonochoristic African cichlid, *Haplochromis burtoni*, increased aggressiveness in relation to increased social dominance was highly correlated with increased expression of GnRH-I (White et al., 2002). Although there is some evidence for a behavioral role for GnRH, the wealth of data regarding GnRH in the POAH indicates a correlation of GnRH expression/cell abundance and gonadal state (Maruska et al., 2007b; Senthilkumaran et al., 1999a).

These two hormones AVT and GnRH are produced in neurons within the brain and are secreted via nerve fibers to act at locations containing receptors specific for that hormone (Mahlmann et al., 1994a; Yu et al., 1998). While a wealth of data exists regarding these hormone systems separately, little is known about linkage between these two systems. Co-occurrence and co-variation of AVT and GnRH have been studied in several fish models (Maruska et al., 2007b; Saito et al., 2003). Neurons and fibers have

been noted to co-occur in the POAH. The rapid actions of exogenous AVT and the corresponding rapid changes that occur in behavioral manipulation experiments indicate that AVT plays a role in early sexual phenotype. In addition, injections of GnRH are known to cause drastic changes in the reproductive state of many vertebrates, especially gamete maturation (Gore, 2002).

A potential route of action between these two hormones could be AVT acting via its receptor on GnRH neurons as has been suggested in chicken (D'Hondt et al., 2000). The fact that these two hormones occur in the same region of the brain and behavioral response linked to AVT appears to occur before gonadal transition associated with GnRH suggests that AVT may act on GnRH neurons to regulate gonadal sex change.

Hermaphroditic fishes that change from one sex to the other are good models to study sexual behavior and related hormonal changes that occur in the brain due to their abilities to reorganize their sexual phenotype as adults. Male sex-specific coloration patterns and behavior are particularly evident in species where males are territorial and guard harems of females such as wrasses and groupers. Sex change from female to male (protogyny) is the predominant form of hermaphroditism in marine teleost fishes (Munoz and Warner, 2003), and in protogynous groupers and wrasses this sex change is dependent on social environment (Liu and Sadovy, 2004b; Mackie, 2003; Semsar and Godwin, 2004b).

In species where a single large male controls a harem of females, removal of the large male results in the largest female of the group assuming male behavior within minutes to hours and a transition to male gonadal sex within days to weeks (Black et al., 2005; Liu and Sadovy, 2004a; Mackie, 2003; Warner and Swearer, 1991a). However, behavioral sex change must occur far quicker than gonadal change if social group order is to be maintained. In the bluehead wrasse, field experiments have shown that behavioral

sex change occurs within hours of dominant male removal (Warner and Swearer, 1991a). In groupers, numerous studies have demonstrated the involvement of sex steroids in the control of gonadal transition (Bhandari et al., 2004; Nakamura et al., 2003; Yeh et al., 2003), and other studies have investigated the roles of external social environment resulting in gonadal sex change (Liu and Sadovy, 2004b; Mackie, 2003). However, information on neuroendocrine control of sex change is currently lacking in any grouper species.

In the sex changing species of wrasse and goby, neural and hormonal control of behavioral sex change has been studied in detail and due to its suggested role in mammalian sexual behavior the AVP/AVT system has received the most attention. In bluehead wrasse, cellular AVT mRNA abundance is higher in terminal phase males when compared to subordinate females (Semsar and Godwin, 2003). This trend is also observed between sexual phenotypes in the gonochoristic fish such as half spotted goby (Maruska et al., 2007b) and Atlantic midshipman (Foran and Bass, 1999a). Behavioral sex and modification of sexual traits such as sound production and courtship behavior are observed within minutes of AVT administration to the brain in the bluehead wrasse (Semsar et al., 2001b), Atlantic midshipman (Goodson and Bass, 2000) and peacock blenny (Carneiro et al., 2003). However, the downstream effects of this hormonal pathway with GnRH have not been described in fish, and the relationship of AVT system with gonadal sex change is unclear.

Recently, two V1a type receptors have been isolated in fish by Lema (2010) and these receptors have a wide distribution in the brain (Balment et al., 2006b; Mahlmann et al., 1994a). Male typical behavior has been linked to this receptor form in the bluehead wrasse. For example, when an antagonist (blocker) specific to the V1a receptor type is administered to sex changing females competing for territories, no dominance over the

territory is established (Semsar et al., 2001b). Similar evidence for the role of AVT is currently lacking in groupers. In addition, there is no information on neural or endocrine targets of AVT within the brain affecting sexual behavior in any fish species. A few studies have suggested GnRH as a good candidate for this action due to its proximity to AVT, its role in mediating gonadal function and potential roles of multiple GnRHs affecting behavior (Bass and Grober, 2001; Maruska et al., 2007a). All studies to date have examined either the effects of external administration of hormones as mediators of sexual behavior or phenotypic variations in abundances of hormones or their production centers in relation to sexual state. The aim of the following research was to test the hypothesis that coordinated interactions between the neuropeptide hormones AVT and GnRH facilitate the process of behavioral and gonadal sex change in protogynous hermaphrodites.

A small grouper species, the rock hind *Epinephelus adscensionis* was selected as an appropriate model for studying behavioral sex change in relation to the AVT and GnRH hormonal systems. It is a hermaphrodite that is common in the Northwestern Gulf of Mexico and its life history and spawning behavior parallel much larger, commercially important grouper species that would be difficult to maintain in an experimental setting. The rock hind is fast growing and matures at a relatively small size (Potts and Manooch, 1995). Four topics were addressed to investigate the relationship between behavior and reproduction: i) rock hind sex change, sexual characteristics and conditions causing sex change to occur in captivity were detailed as a basis for examining the AVT system and GnRH during this process, ii) the distribution of a vasotocin V1a type receptor identified in rock hind brain was examined for the first time in a fish species using a custom designed antibody then the receptor protein was co-localized with GnRH producing cells within the brain to confirm that a pathway exists for AVT action on GnRH, iii) levels of

AVT, AVT receptors, and GnRH messenger RNAs (mRNAs) were compared between male and female rock hind phenotypes, and iv) female rock hind at early stages of sex change were compared for brain mRNA expression of AVT, AVT receptors, and GnRH to determine the order of hormonal change during the process of sexual inversion in this species.

Chapter 2: Behavior, Color Change and Time for Sexual Inversion in a Protogynous Grouper *Epinephelus adscensionis*

ABSTRACT

Hermaphroditism, associated with territoriality and dominance behavior, is common in the marine environment. While male sex-specific coloration patterns have been documented in groupers, particularly during the spawning season, few data regarding social structure and the context for these color displays are available. In the present study, we define the social structure and male typical behavior of wild rock hind (*Epinephelus adscensionis*). In addition, we detail the captive conditions and time period necessary to induce the onset of the sex-specific coloration and sexual change. Rock hind social group size and typical male social behavior were documented at six oil production platform locations in the Gulf of Mexico. We observed a rapid temporary color display in rock hind that could be turned on and off within three seconds and was used for confronting territory intruders and displays of aggression towards females. The male-specific "tuxedo" pattern consists of a bright yellow tail, a body with alternating dark brown and white patches and a dark bar extending from the upper mandible to the operculum. Identification and size ranges of male, female and intersex fish collected from oil platforms were determined in conjunction with gonadal histology. Rock hind social order is haremic with one dominant male defending a territory and a linear dominance hierarchy among individuals. In five captive behavioral experiments, the largest remaining female rock hind displayed the male specific color pattern within 32d after dominant male removal from the social group. To our knowledge, this is the first evidence in a grouper species of color patterning used to display territoriality and dominance outside of spawning aggregations. The behavioral paradigm described here is

a key component for determining the timing and hormonal modulation involved in this complex sex change process.

INTRODUCTION

Hermaphroditism, associated with territoriality and dominance behavior are common in the marine environment, especially with reef or substrate-associated fish such as groupers and basslets (Serranidae), wrasses (Labridae), and parrotfishes (Scaridae). Dominance hierarchies have been described in several species and are common in protogynous hermaphrodites that change sex from female to male, often resulting in the largest fish in a social group being a dominant and highly aggressive male (Robertson 1972; Shapiro 1981a; Warner & Swearer 1991).

Within territorial protogynous hermaphrodites, wrasses (Labridae) have been extensively studied in terms of behavior and sex change (Robertson 1972; Warner 1982; Semsar & Godwin 2004; Sakai et al. 2007) and the dominance hierarchy has been well described (Warner & Swearer 1991). Typically a single dominant male with a permanent distinctive color pattern controls a harem of females that can greatly vary in number depending on population size and available reef habitat Aggression from the dominant male is typically directed at territorial intruders and the largest female in the harem. This is presumed to occur because the largest female fish is almost always the next in line to change sex (Robertson 1972; Warner 1982; Ross et al. 1983; Lntnesky 1994; Liu & Sadovy 2004).

Within a social group, sex-specific coloration differences in the dominant male have been documented in many species (Robertson 1972; Shapiro 1981a; Warner 1982; Thresher 1984; Lutnesky 1988; Warner & Swearer 1991; Sakai et al. 2003). While male sex-specific coloration patterns are present in many vertebrate classes including

mammals (Bradley & Mundy 2008), birds (Burns 1998), amphibians (Todd & Davis 2007) and fish (Maan et al. 2008) these patterns are particularly evident in protogynous fish species such as parrotfishes (Sparidae), wrasses (Cardwell & Liley 1991; Warner & Swearer 1991; Sakai et al. 2007) and groupers (Serranidae, *Epinephelinae*) (Colin et al. 1987; Sadovy et al. 1994). This sexually distinct color patterning is most prevalent during the spawning season and especially during the day of spawning (Thresher 1984; Cardwell & Liley 1991) and differs from permanent sexually dimorphic patterning seen in other species (Shapiro 1981a; Warner & Swearer 1991; Dawkins & Guilford 1993). Several species of grouper are documented to display distinctive male coloration patterns around the time the time of spawning and in aggregations including the red hind *(Epinephelus guttatus*), rock hind (*E. adscensionis*), scamp (*Mycteroperca phenax*), gag (*M. microlepis*), coney (*Cephalopholis fulva*), tiger (*M. tigris)*, and black groupers (*M. bonaci)* (Colin et al. 1987; Gilmore & Jones 1992). In contrast to ephemeral coloration patterns, these coloration patterns persist even after capture and hours after death in some species (Colin et al. 1987; Sadovy et al. 1994). In a few grouper species, ephemeral coloration patterns have been noted but these are only documented very near the time of gamete release (Thresher 1984; Gilmore & Jones 1992; Sadovy et al. 1994). Blue head wrasses also exhibit variations in color patterning in males in terms of permanent pigmentation patterns as well as short term variation between "opalescent" spawning coloration and deeper hued coloration patterned associated with territorial defense (Dawkins & Guilford 1993) but similar data are not available for the rock hind. An excellent method to study these behavioral changes in protogynous fishes has been through male removal experiments (Robertson 1972; Warner & Swearer 1991; Mackie 2003; Semsar & Godwin 2004).

Male removal experiments have been conducted in field and laboratory settings to determine the conditions and time duration to induce sex change in several protogynous species. Typically, two major changes occur during these experiments: 1) the onset of male-like behavior and aggression; and 2) transition of the gonad from ovary to testis. In the wrasses and gobies (Gobiidae), behavioral sex change is rapid and can occur within minutes to hours of dominant male removal in the largest remaining fish (Warner & Swearer 1991; Godwin 2009). In groupers, field and laboratory studies have been conducted to determine the time necessary for gonadal change ranging from three to seven weeks (Bhandari et al. 2003; Liu & Sadovy 2004). Gonadal sex change takes considerably longer than behavioral change due to the need to reorganize the gonad morphologically and in terms of steroidogenic capacity. In bluehead wrasse, this process takes as few as eight days (Warner & Swearer 1991). In grouper species, gonadal transition from ovary to testis occurs within three weeks in the half-moon grouper (*Epinephelus rivulatus*) (Mackie 2003) and up to seven weeks in the chocolate hind (*Cephalopholis boenak)* (Liu & Sadovy 2004). However, information on any concurrent behavioral changes as demonstrated in the bluehead wrasse (Warner & Swearer 1991) and Anthias (*Anthias squamipinnis)* (Shapiro 1981b) are lacking in any grouper species.

The focus of this study is the rock hind, a protogynous grouper that has a wide geographic distribution from Ascension Island in the Eastern Atlantic to North Carolina in the Western Atlantic and throughout the Caribbean and the Gulf of Mexico (Polovina & Ralston 1987). The rock hind is an Epinepheline species that is relatively abundant on oil and gas production platforms in the Gulf of Mexico and is observed at depths accessible by SCUBA divers (Hastings et al. 1976; Rooker et al. 1997). In a brief description concerning spawning aggregations of several grouper species by Colin

(1987), rock hind males were reported to be sexually dichromatic, having black and white blotches in the context of spawning and courtship.

Here we present behavioral observations of the social structure and dramatic, temporary sex-specific color displays in wild rock hind. Furthermore, we present experimental data on the conditions and time period necessary to induce the onset of the sex-specific coloration and sex change in captive fish maintained in the laboratory.

METHODS

Field observations of rock hind social organization and color patterning were made on SCUBA at depths from 6-15 m on oil and gas production platform habitats in the Gulf of Mexico near Port Aransas, Texas (Fig. 1a). Production platforms in the Gulf of Mexico have been in place for 20 years or more (Hastings et al. 1976) and typically have considerable overgrowth of encrusting organisms such as barnacles, sponges and corals, as well as abundant fish populations (Hastings et al. 1976) (Fig. 1b). Collections for sex identification and captive study of rock hind behavior were also made on platforms in the Gulf of Mexico on SCUBA using spear fishing and underwater hook and line fishing. For captive experiments, fish were transported to facilities at the University of Texas at Austin Marine Science Institute, Port Aransas, TX USA. All fish used in this study were treated according to a protocol approved by the University of Texas Animal Care and Use Committee.

Field observations of rock hind social behavior were made on 20 social groups at six platform locations in the Gulf of Mexico from March to November 2008. Rock hind behavior was observed by divers on SCUBA and from supplemental underwater videos. Assessments of rock hind social group size were recorded along with coloration patterns and distance between territories. After observations, rock hind that were previously

observed in male-typical display as well as other fish in the vicinity of each social group were collected for sex determination by spear fishing and underwater hook and line fishing. On the boat, rock hind were anesthetized with an overdose of clove oil (0.1ml/l) and measured for total length. Fish were killed by severing the spinal column and gonadal tissue was removed and preserved in 10% buffered formalin until further processing.

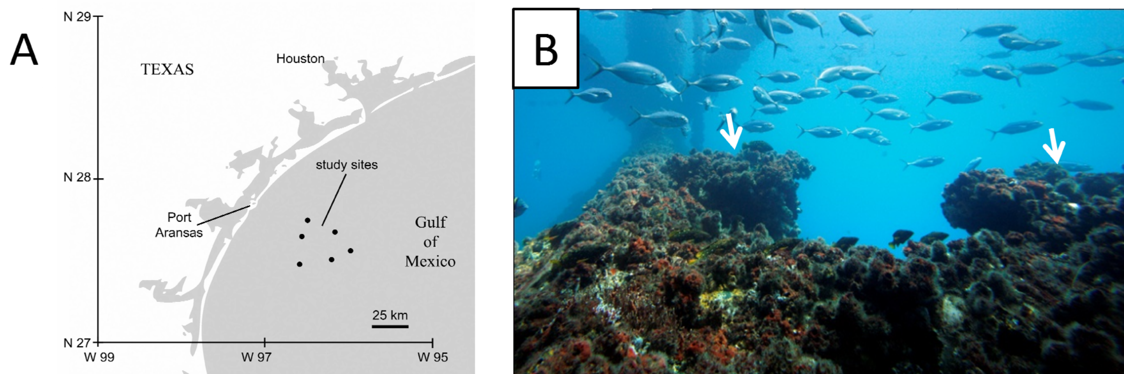

Figure 2.1. A) Map of Texas coast showing oil platform study and sampling sites (black dots). B) Picture of oil platform support rung with heavy barnacle growth showing two adjacent rock hind territories (white arrows).

Gonadal tissue was rinsed then cryoprotected in 30% sucrose solution followed by embedding in mounting media (OCT Compound, Sakura, Torrance, CA). Fifteen micron cross-sections were made on a cryostat and thaw mounted on slides (Superfrost plus, Erie Scientific). Sections were air dried then rehydrated in PBS (20 mM phosphate base, 150 mM NaCl, pH 7.6) and stained using Myers' Hematoxylin and Eosin. After staining, sections were dehydrated in an alcohol series and cleared with xylene. Slides were cover-slipped with Cytoseal-60 (Richard-Allan, Kalamazoo, MI) and allowed to dry overnight. Representative sections were imaged on a Nikon eclipse microscope with

black and white high resolution camera (CoolSnap cf mono). Sex determined from gonadal sections was compared to behavior and color patterning recorded in focal fish before capture and to size recorded after capture.

Rock hind behavioral manipulation experiments

Thirty rock hind were captured from oil platform locations in April and May for use in captive experiments to determine the time course of behavioral and occurrence of gonadal sex change. Fish were placed in a dechlorinated fresh water bath for 2 minutes to remove ectoparasites (Noga 2000) then held together in a 3m diameter tank for one week to recover from capture and transport. Captive conditions were 26 °C temperature and 12.5:11.5 light:dark photoperiod for all experiments. Fish were fed a mixture of sardines, shrimp and squid to satiation every day. After the recovery period, fish were anesthetized and examined for sexual condition by applying pressure on the ventral side to collect milt or by inserting a small cannula into the oviduct to collect a biopsy sample. Biopsy samples were observed under the microscope following the methods of Neidig (2000).

Fish were divided among five tanks in which one male, confirmed by expressing milt (290-320mm), one medium sized female (260-280mm), and three smaller females (180-250mm), were placed into each group (confirmed from biopsy samples). Fish in which the sex could not be definitively determined were excluded from the experiments. A single habitat structure made of three 0.5m sections of 10 cm diameter PVC pipe stacked in a pyramid configuration and held together with plastic cable ties was placed into each 1.2m depth x 3m diameter tank. Five rock hind social groups with identical habitats were set up for the experiments.

Three weeks were allowed for the development of social hierarchies; the characteristic color display in each male (determined in the field studies) was noted

before any behavioral manipulations were made. After verification of male patterning, the male from each tank was removed. Daily visual and video observations of the focal fish (the largest female remaining after male removal) were made for seven weeks for 1.5 h near the end of each day when the probability of observing male behavior was highest according to preliminary observations. Observations were scored for onset of aggression, behavioral changes and the appearance of the characteristic male color pattern. Data from the five replicated experiments were compared for major changes in behavior and onset of male typical behavior profiles. Mean and standard error were calculated for time from male removal to: first observation of aggression in the largest remaining fish, first change in coloration and onset of full color display by the dominant fish. A timeline for rock hind behavioral sex change was constructed.

RESULTS

Field observations

The typical color pattern of both male and female rock hinds is a cryptic camouflage pattern consisting of white, light brown and red-brown spots (Figure 2A). When agitated, both male and female rock hind can display a darkened horizontal bar extending from the upper mandible through the eye and ending at the operculum (Fig. 2B). However, the male also displays a dramatic, temporary color pattern that we designated the “tuxedo” pattern (Figure 2C-D) consisting of a bright yellow tail, and a body with alternating dark brown and white patches. In addition, a distinctive pattern was displayed on the head consisting of a dark bar extending from the upper mandible to the operculum and a bright white spot below the eye (Fig. 2D). Although not specifically measured, this pattern appeared to be very consistent from fish to fish in field observations.

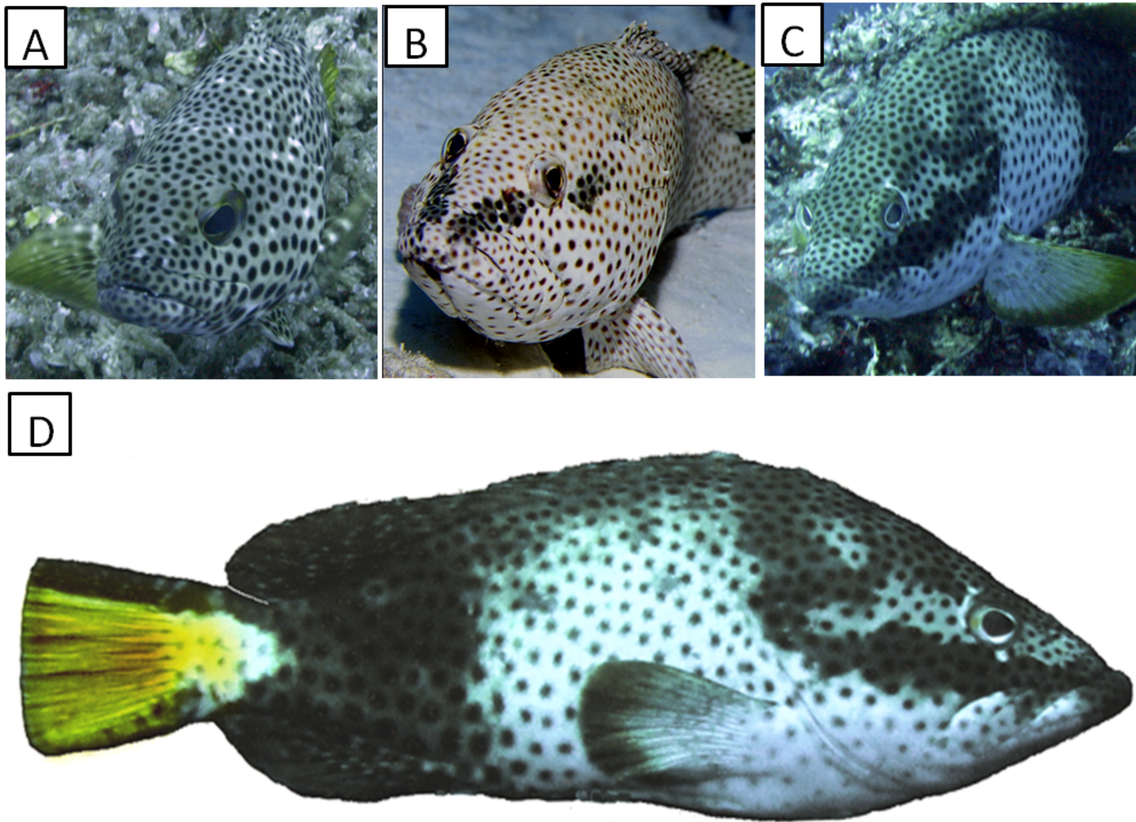

Figure 2.2. Photographs showing head markings of rock hind A) male or female cryptic pattern; B) male or female aggressive pattern; C) male-specific territorial “tuxedo” pattern; and D) Aspects of the “tuxedo” pattern showing white and brown patches, yellow tail with brown border and head markings.

Male rock hind were observed guarding territories during all months studied, and this tuxedo pattern was displayed when confronting potential territory intruders. It was also observed when males were displaying aggression towards the females in the harem, especially the largest ones. This color change from camouflage to tuxedo pattern was quite fast and could be turned “on” or “off” within 3 seconds. Accompanying the tuxedo pattern was a characteristic “head shake”, a lateral side to side movement of 3-5 seconds that was directed towards females within the harem or to territorial intruders. The sex of ten individuals that were observed displaying the tuxedo pattern was verified

histologically by the presence of functional testis and no evidence of ovarian tissue in the gonads of these fish (Fig 4B); although in three cases yellow-brown bodies were noted in gonadal sections, potentially indicating remnants of atretic oocytes and recent sex change (Sadovy & Shapiro 1987).

Rock hind Social Behavior

Rock hind social order was observed to be haremic with one dominant male defending a territory and several smaller females residing within that territory. A linear dominance hierarchy was observed in many cases with one or two larger female(s) holding sub-territories and displaying aggressive behavior (chasing, biting and eye-bar display) to smaller females.

Rock hind behavior and harem size appeared to depend on the size and quality of the available habitat in the field. For example, on offshore pinnacle reefs approximately 6m in diameter, a single territory and harem was observed on each mound with 10m of vertical relief. In contrast, production platforms had a three dimensional lattice of structures with support rungs spaced every 10m of depth, and several social groups could be observed on each rung if sufficient barnacle or sponge growth was present. Territory spacing was also dependant on habitat composition and typical spacing ranged 6 to 10m; however with sufficient structure, only 3m of spacing between territories was observed at one site.

Sex Ratio

Rock hind social group size observed in twenty social groups over six sites ranged from five to nine individuals with a mean of 7.4±0.3 individuals, based on 30 minute observations of each group and proximity to defined territory of the displaying male near a specific hole or crevice that the focal male occupied. This would equate to a mean sex

ratio of 6.4 females to one male in the six locations observed. Solitary tuxedo displaying males were observed at three sites. These fish occupied small areas with little structure and no subordinate fish present. Occasionally they were observed roving into territories and were chased away by the dominant male of that territory.

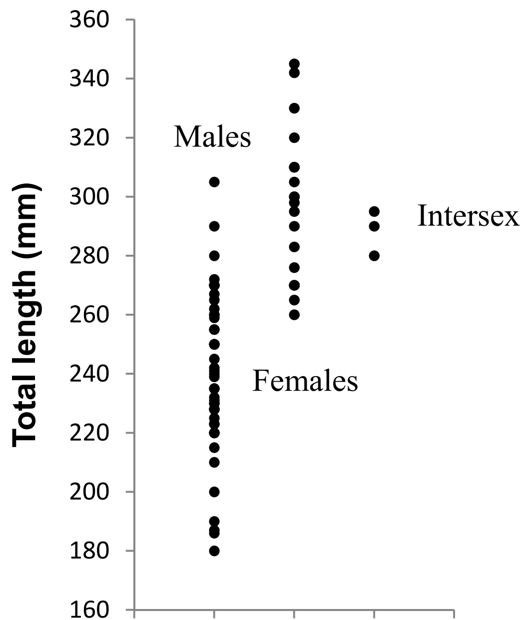

Figure 2.3. Length distribution of female, male and intersex rock hind captured at 6 oil platform study sites in the Gulf of Mexico during March through November 2008. Total n = 68.

Gonadal histology

Of the 68 rock hind sampled from platforms in the Gulf of Mexico, 46 were female with mean TL 240±4 mm; 19 were identified as male with mean TL 297±6 mm and 3 fish were intersex with mean TL 288±4 mm. Mean male and intersex TL was significantly different from female TL (ANOVA with Tukey comparison $p<0.001$ and $p=0.009$ for F vs. M and F vs. intersex, respectively). A size at length diagram is

presented in Figure 3, demonstrating the size ranges observed in sampled fish. Overlap was observed between males and females at 260 to 305mm, and no males were captured below this size range.

Mature female rock hind were observed ranging in size from 180mm to 305mm. Ovarian tissue was observed in several stages of development from previtellogenic to late stage atretic oocytes, with the most common being previtellogenic oocytes (Fig. 4A), however no females were captured with ripe ovaries, indicating that they were captured outside the spawning period. Males were also observed in several stages of development, with the majority of testis having spermatocytes and spermatids present (Fig. 4B). Three intersex fish were identified in the field studies. On gross examination, these fish had reduced gonad size and the gonads appeared dark in color compared to typical ovaries and testis. In cross-section, all three intersex fish had atretic previtellogenic oocytes, dense connective tissue and testicular tissue in the early stages of development, with numerous crypts of spermatocytes and few crypts of spermatids (Fig. 4C).

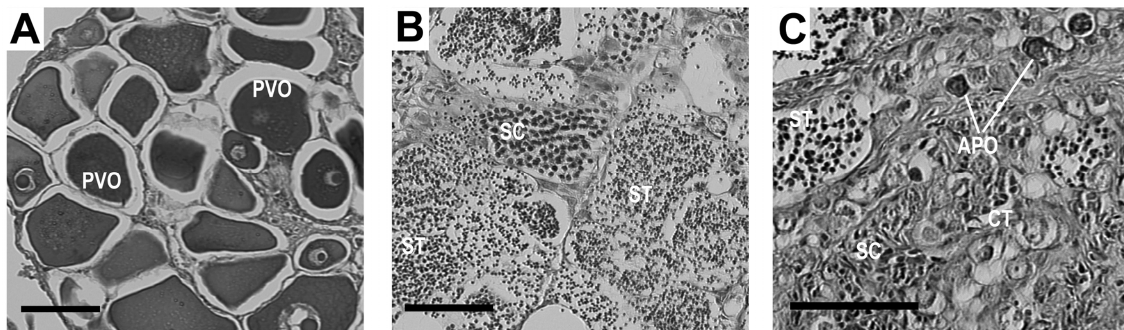

Figure 2.4. Representative cross sections of A) female, B) male, and C) intersex gonadal tissue from rock hind captured on oil platform habitats in the Gulf of Mexico. PVO: previtellogenic oocytes; SC: spermatocytes; ST: spermatids; APO: atretic previtellogenic oocytes; and CT: connective tissue. Scale bars = 50µm.

Behavioral experiments in captive fish:

Before male removal, observations revealed the presence of a single dominant male that would occupy the tube structure and periodically rush at females that had entered the structure causing them to flee. This behavior was repeated many times during each observation. In all social groups the largest female also had a defined territory on one side of the structure near an opening and would prevent entry of any of the smaller females into this space. The male would "patrol" and occasionally flash the tuxedo pattern while swimming around the entire tank. During these patrols the male would sometimes displace the largest female from her territory and occupy it or in some cases, physically push the female out of the way with his snout. This behavior was most often observed during the last hours of light and was prevalent throughout the day before spawning in experiments as described below.

A timeline for rock hind behavioral change after male removal is shown in Figure 5. In the male removal experiments, the remaining fish would typically hide for one day post male removal and little activity was observed during this period. Increased patrolling of the entire tank and a partial tuxedo display was observed by 21±4d in the largest female. The partial display showed the tuxedo body coloration and yellow tail but no prominent dark bar below the eye. The timing of the onset of a complete tuxedo display was remarkably consistent occurring at 32±2d in all 5 groups.

In two of the groups, the recently sex-changed fish successfully fertilized eggs at 42 and 67d respectively. Fertilization was verified by following the egg development over 24 hours. After spawning, females had numerous bite marks on both flanks, apparently from the recently sex-changed male. Fish from this experiment were used in other studies and were not checked for gonadal state immediately after the experiment. However, all of the largest fish from the three groups that did not spawn were verified as

males by applying pressure to the abdomen and expressing milt. All remaining fish from the experiment were later confirmed to be females by gonadal biopsy.

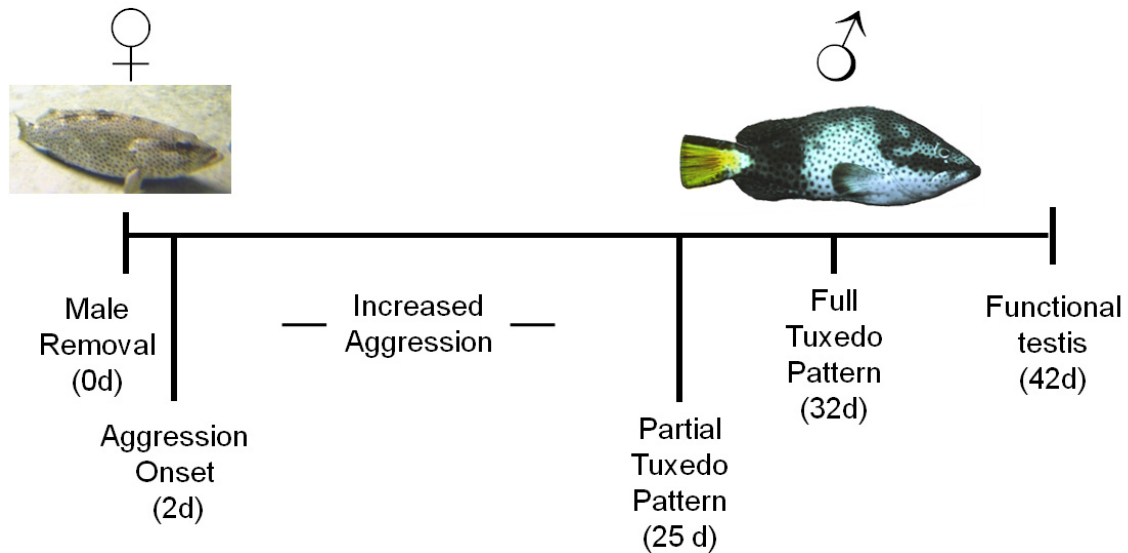

Figure 2.5. Timeline for attainment of male specific tuxedo color display in rock hind based on captive observations in five replicated experiments. Captive conditions consisted of one habitat structure and starting conditions of one male, one intermediate and three smaller rock hind in each 3m diameter tank.

DISCUSSION

Sex-specific color patterning, observed in many reef-associated species during the spawning season, is usually reported as permanent, remaining present for hours to days after death in the bluehead wrasse (Semsar & Godwin 2004) and groupers (Colin et al. 1987). However, the intricate and striking tuxedo pattern reported here in rock hind is temporary, can be turned on and off very quickly, and is not present after capture or after death. To our knowledge, this is the first evidence of color patterning in a grouper species used for display of territorial defense and dominance except for during spawning

aggregations. The rapid change in color patterning reported here is likely under neural control as reported in cichlid (*Astatotilapia burtoni)* (Muske & Fernald 1987a).

The behavior and social structure observed in rock hind on offshore platforms is similar to that reported for the half-moon grouper on reefs in Australia (Mackie 2003). The male to female sex ratio of 1:6.4 found in this study is also similar to that seen in half-moon grouper (1:6). In addition, Mackie (2003) reported the presence of bachelor males at the reef study site similar to that reported here. Although he described spermatogenic tissue within the ovaries of many females, this was not observed in rock hind ovaries in this study; however Mackie sampled during the spawning season and many females had well-developed ovaries. In the present study, none of the gonads examined were well developed and the fish were apparently collected outside of the spawning season.

Rock hind displayed a linear social hierarchy similar to that reported in cleaner wrasse (Robertson 1972). In the field and laboratory experiments, rock hind males were most aggressive toward the largest females that held sub-territories and larger females were most aggressive toward small females. This selective aggression by the male was presumed by Robertson (1972) in wrasse to be because the largest female is next in line to change sex. This behavior in rock hind was highlighted in some preliminary experiments with mirrors whereupon seeing a reflection, rock hind would attempt to chase a similar-sized image even when other sized fish were present in the reflection (R. Kline, unpublished data).

No male or intersex rock hind were observed below the size of 260mm total length in the field collections, suggesting that rock hind have a minimum size required to transition to male. However, determination of minimum size for rock hind sex change was not the focus of this study, and a wide range of male sizes is reported in large fishery

studies of groupers in different habitats (Mackie 2003; Liu & Sadovy 2004). It is likely that smaller male rock hind do occur and this topic warrants further study. Direct determination of sex by examination of biopsy samples (this study; also see Mackie 2003) or reliable secondary sex characteristics (Liu & Sadovy 2004) is advised in behavioral experiments with small groupers because individuals captured from several sites cannot be assigned a sex identification by relative size alone. In general, we observed that rock hind males were larger than females among neighboring territories. However, even at greater depths at the same site and from site to site, females could be larger than some males captured at other locations.

Although the gonadal state of males at the onset of color pattering in behavioral experiments was not investigated here, participation in spawning activity by two fish in captivity within a few weeks after they first displayed the tuxedo pattern, provides compelling evidence that these fish were at least intersex if not fully sex-changed males by 32±2d. Furthermore, the field data support this by the fact that all tuxedo-displaying males sampled from the field were identified as male by gonadal histology. Other studies in grouper show that the half-moon grouper transition to producing mature sperm by three weeks in many cases (Mackie 2003) and seven weeks in the chocolate hind (Liu & Sadovy 2004). Interestingly, the time required for induction of sex change with steroid implants in groupers (Yeh et al. 2003; Kline et al. 2008) is similar to that reported here for behavioral experiments, suggesting that the change in steroid profiles are initiated quickly after male removal in order to transition the gonad within this time period.

Aside from the solitary males noted, only one male was observed within each social group at the platform sites. Sneaker males that look and behave like females within the social group have been described in haremic and lek-based mating systems in the gonochoristic midshipman (*Porichthys notatus*)(Brantley & Bass 1994), and protogynous

bluehead wrasse (Warner & Schultz 1992). However sneaker males have not been described in groupers and were not observed in this study. During spawning, smaller male groupers can streak in and release gametes during pair spawning of a larger male in the tiger grouper (Sadovy et al. 1994); however, these fish look and behave similarly to other males in the area. It is possible that these solitary males function as a form of "cheater" male in rock hind as well, and in this scenario it would be beneficial to remain male and streak in to spawn or take over a recently vacant territory rather than change back to female. It is unclear if rock hind can change back from male to female as described for the chocolate hind (Liu & Sadovy 2004). Although this condition might be artificially induced, the presence of bachelor males occupying sub-optimal habitat and the relatively long period of sex change reported here suggest that it is unlikely to occur in the wild rock hind populations observed in this study.

Although solitary "tuxedo" displaying males were seen in wild rock hind populations, their presence was intentionally avoided in the captive sex change studies reported here. In wild populations, they likely play an important role in the social dynamics of rock hind harems when the resident dominant male is lost. Territory-holding half-moon grouper males removed from reefs by Mackie (2003) in Australia were replaced by solitary immigrant males in 45% of the cases. Curiously, after the immigrant males were removed, smaller remaining fish did not move in to replace them, suggesting a minimum size is necessary. The importance of these solitary males and how it affects the sex change process in the largest remaining female in wild populations of rock hind warrants further study.

Color displays, especially the dark band below the eye in the tuxedo pattern, appears very important to the male display. The presence of an eye-bar has been extensively studied in the gonochoristic cichlid, and it is only displayed in dominant

males (Muske & Fernald 1987b). In rock hind, both sexes can display a bar through the eye. However, the male specific tuxedo pattern appears to be a chromatically opposite pattern to the aggressive eye-bar pattern that both males and females can display (see Figure 2b vs. 2c). When captive rock hind males are shown the tuxedo pattern on a video monitor, they react very strongly to it in comparison to images without the tuxedo bar on the face (R. Kline, unpublished data). It is also possible that other components of this territorial visual display are not visible to the human eye and occur in the UV spectrum as seen in the damselfish (*Pomacentrus amboinensis*) (Siebeck 2004).

CONCLUSIONS

Rock hind is a protogynous hermaphrodite that uses a striking and temporary "tuxedo" display in association with aggression and dominance and defense of territories. In behavioral experiments, female rock hind displayed the male specific "tuxedo" color pattern within 32d of dominant male removal from the social group. This is the first evidence reported for groupers of temporary color patterning used for display of territoriality and aggression outside of the spawning season. A key result of this study is the establishment of an effective experimental paradigm for inducing sex change under captive, controlled conditions and that is an important advance enabling mechanistic studies of this complex sex change process.

Chapter 3: Immunohistochemical Localization of the AVT V1a2 Receptor in the Brain of a Sex Changing Fish *Epinephelus adscensionis*

ABSTRACT

The present study describes the distribution of the arginine vasotocin (AVT) V1a2 receptor subtype (AVTr) throughout the brain of a sex changing grouper, rock hind *Epinephelus adscensionis*. The objectives of this study were to map AVTr brain expression for potential linkages of the AVT hormone system with sex-specific behaviors observed in this fish and to examine sex-specific differences that might exist. An antibody was designed for rock hind AVTr against the deduced amino acid sequence for the third intracellular loop identified expression limited to cell bodies throughout the brain and not present in neuronal fibers. AVTr was widely distributed throughout the brain indicating that AVT may act as a neuromodulator via this V1a receptor subtype. AVTr was present in regions associated with behavior, reproduction and spatial learning, as well as sensory functions such as vision, olfaction and lateral line sensory processing. We observed the highest AVTr expression in granular cell formations in the internal cellular layer of olfactory bulbs, torus longitudinalis, granular cerebellum, valvula of the cerebellum, nuclei of the lateral and posterior recesses, and granular eminence. High expression was also observed in the preoptic area, anterior hypothalamus, and habenular nucleus. No obvious sex differences were noted in any region of the rock hind brain.

INTRODUCTION

Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) influence many behavioral and physiological processes such as sexual and social behaviors, seasonal and circadian rhythms, stress response as well as metabolic, cardiovascular and osmotic processes (see review by Balment et al., 2006). Major

functions of the AVT/AVP system in the brain include control of behaviors such as social approach, courtship and aggression, with these behaviors typically attributed to males. Most notably, research with voles has revealed interspecies differences in monogamous and polygamous species attributed to differential expression of the AVP V1a receptor in specific brain regions (Insel et al., 1994; Nair and Young, 2006).

The AVP system in mammals has received the most attention and three receptor subtypes have been identified that differ in their tissue distribution and pharmacological characterization. The three receptors designated V1a, V1b and V2, are membrane associated, G protein-coupled receptors that are expressed in numerous tissues throughout the body with considerable overlap (Birnbaumer, 2000). However, differences are seen in their function through second messenger systems, and major effects are discerned through pharmacological experiments. There is a clear linkage between V1a receptor expression in the brain and sex specific behaviors in mammals, birds, amphibians and fish (Baeyens and Cornett, 2006; Hasunuma et al., 2007; Insel et al., 1994; Semsar et al., 2001), while the V1b receptor mediates the vasopressor response through an action in the pituitary where it is linked with adrenocorticotropic hormone (ACTH) function (Jurkevich et al., 2005; Tanoue et al., 2004). The V2 receptor, on the other hand, regulates water uptake via aquaporins in the kidney (Hayashi et al., 1994). Both V1 receptor forms utilize the inositol phosphate second messenger system and mobilize intracellular Ca^{2+} whereas the V2 subtype is linked to the adenylate cyclase pathway (Birnbaumer, 2000). In mammals, the V1 type receptors can be further distinguished from one another based on differing agonist and antagonist affinities (Manning and Sawyer, 1993; Serradeil-Le Gal et al., 2002). However, similar research on specific AVT receptor agonists and antagonists is currently lacking for non-mammalian vertebrates.

Although the three major subtypes have been well characterized in mammals, the function and presence of homologous receptors is less clear in fish. Mahlmann et al. (1994) first isolated an AVT receptor in white sucker and characterized it as pharmacologically and functionally similar to the mammalian V1a type receptor. Other investigators have shown the V1 type receptor associated with osmoregulation in the kidney and widely distributed in other tissues such as brain, pituitary and gill (Lema, 2010; Warne, 2001).

Recently, three forms of AVT receptor mRNA were isolated in newt and identified by amino acid homologies and mRNA tissue distribution as V1a, V1b and V2 subtype receptors (Hasunuma et al., 2007). The two proposed V1 subtypes were highly expressed in the brain and pituitary whereas the proposed V2 subtype was highly expressed in the kidney but absent from the pituitary, similar to the distribution of the AVP system in mammals (Birnbaumer, 2000; Ostrowski et al., 1994). In pupfish, Lema (2010) isolated mRNA sequences for three AVT receptor subtypes and identified them by their mRNA tissue distribution and amino acid homologies as V1a1, V1a2 and V2 receptors. He reported the presence of two distinct forms of the V1a subtype, with overlapping mRNA distributions in the forebrain, midbrain, cerebellum and hindbrain. This study did not identify a V1b receptor in pupfish, although its existence cannot be discounted based on the data presented.

The presence of three receptor subtypes in teleost fish and their range of functions in relation to their mammalian counterparts are unclear. Numerous effects of AVT have been noted and some similar functions have been attributed to receptor subtypes as characterized in mammals such as changes in social behavior (Semsar et al., 2001), osmoregulation (Warne et al., 2005) and smooth muscle contraction (Conklin et al., 1999). Behavior experiments in fish and amphibians have implicated a V1a type receptor

based on the effects of an AVP V1a antagonist, Manning compound. With these behavioral effects attributed to the V1a receptor and the recent identification of multiple V1a type receptors by Lema (2010) with overlapping expression in the brain, there is a need for localization studies to understand receptor distribution and to infer potential function(s).

Neuroanatomical distribution of AVT binding sites has been described in only a few fish and amphibian species. In one study, fluorescently labeled AVT was administered directly to the brain of newt and specific cell populations in the reticular formation of the hindbrain were labeled and identified as areas of AVT action (Lewis et al., 2005). Similarly, only one study in fish has reported identification of AVT binding sites in the pituitary and several generalized brain regions in the seabass, Dicentrarchus labrax (Moons et al. (1989). However, the receptor subtypes and detailed examination in specific neuronal populations could not be established using this method.

In the only published immunohistochemistry (IHC) study of an AVT receptor in fish, Warne (2001) cloned and characterized a V1a type receptor and developed an antibody against a 15 amino acid sequence of the third intracellular loop (Warne et al., 2005). This study focused on osmoregulation in the European flounder, Platichthys flesus and IHC experiments with the AVTr V1a antibody revealed strong staining in kidney tissue as well as functional evidence of its role in osmoregulation. Though this study noted mRNA expression of the V1a receptor in the brain tissue, no information regarding its distribution in specific brain regions was provided.

As in mammals, sex specific behavior has been documented in several fish species and correlated with changes in AVT hormone expression in specific cell populations in the preoptic area-anterior hypothalamus (POAH). This difference has been documented in several species of sex changing fish such as wrasse, goby, and anemone

fish where the sex specific behavior has been correlated with AVT cell populations in the POAH that vary in relation to sexual state (Godwin et al., 2000; Grober and Sunobe, 1996; Iwata et al., 2008) . The AVT V1a type has been implicated in the control of certain male behaviors in different vertebrate groups because administration of an AVP V1a specific antagonist can disrupt these behaviors. The reported effects range from delayed onset of aggression and marking behavior in mammals (Albers et al., 1986; Winslow et al., 1993), reduced courtship and aggression in fish (Semsar et al., 2001) to the inhibition of clasping behavior in amphibians (Moore and Miller, 1983).

Aside from the effects of AVP V1a antagonists and sexual dimorphism of specific AVT producing cells types and a single published study on AVT binding sites listed above, little is known regarding the function and distribution of vasotocin receptors in the brain of fish. The objective of this research was to describe the distribution of the AVT V1a receptor using an antibody designed against the deduced amino acid sequence from the AVT V1a2 receptor cDNA sequence in rock hind. Rock hind behavior and gonadal sex can be altered via manipulations of the social environment (Chapter 2) and ongoing research on sex specific behaviors and AVT prompted this study on the distribution of the AVT V1a receptor in this species. Of particular interest are the sites of action in the brain, especially the POAH and other areas that might affect sexual behavior and have potential for downstream effects on the reproductive system.

METHODS

Animals

Seven rock hind were captured from oil platforms in the Gulf of Mexico near Port Aransas, TX in August, 2008 and used immediately for IHC and Western blot studies. Sex was determined by gross examination of the gonads after the collection of brain

samples for IHC. Microscopic examination showed that these fish were not in reproductive season with testis and ovaries in the early stages of development. All rock hind used in this study were treated in compliance with a protocol approved by the University of Texas at Austin Animal Care and Use Committee.

Antibody	Antigen	Supplier	Host	Type
RH V1a2	IKYKKRKSTAGAANK Rock hind V1a2 3rd intracellular loop	Alpha Diagnostics	Rabbit	Polyclonal

Table 3.1. Information for the rock hind V1a2 receptor antibody used in this study.

Antibodies to the rock hind V1a2 subtype vasotocin receptor were raised in rabbit against a region corresponding to the predicted protein sequence (GenBank accession no. HQ141396) of the third intracellular loop of the receptor and affinity purified (Table 1). This sequence has highest similarity to the AVT V1a2 (GenBank accession no. GQ981412) receptor as identified in pupfish by Lema (2010). The two forms of the V1a recently published in Genbank for rock hind differ substantially in the antigenic site of the third intracellular loop (Fig. 1). Although, no other AVT receptor forms are available for rock hind and no V1b from has been identified in any fish, the third intracellular loop region differs between V1a and V1b receptor forms in newt (Hasunuma et al., 2007).

To test the specificity of the AVTr antibody, western blot was performed to determine which proteins were identified by the antibody. One male and one female rock hind were killed by an overdose of MS-222 (1 g l-1) and whole brain removed. Brain tissue was extracted using the Q-proteome cell compartment extraction kit to isolate membrane, cytosolic and nuclear fractions (Invitrogen USA). Membrane protein (15 µg) from each cell compartment fraction was loaded and run on a 10% sodium dodecyl

sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel. Following overnight transfer to Polyvinylidene fluoride (PVDF) membrane, the membranes were washed 3 times for 5 minutes with PBS-T (20 mM phosphate base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and immersed in blocking buffer (5% normal goat serum and 0.5% porcine gelatin in PBS-T) for one hour at room temperature. Membranes were then rinsed in PBS-T and incubated overnight at 4° C with AVTr antibody or antibody pre-absorbed overnight with 1 μg of blocking peptide to 1 μl antibody at a final dilution of 1:1000 in PBS-T. Following primary antibody incubation, membranes were washed 3 X 5 min with PBS-T and incubated with secondary HRP linked goat anti-rabbit antibody (AbCam, Cambridge, MA) at a final concentration of 1:5,000 in PBS-T with 5% nonfat milk for two hours at room temperature. Membranes were again washed 3 X 5 minutes with PBS-T, and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) was applied to visualize AVTr on ECL hyperfilm (Amersham, Piscataway, NJ).

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V1a2 Rock hind (HQ141396) PVVILVMCYGFICHSIWNNIKYKKRKSTAGAANKNGLIGKHSVSSIT--247
V1a2 Fugu      (AY027887) PVTMLMMCYGFICHSIWKNIKFKKRKGTTGAATKNGLIGKNSVSSIT--274
V1a2 Pupfish   (GQ981413  PVVILILCYGFICHSIWKNIKYKKKKTVAGAAGKNGLIGKCSVSSIT--248
V1a1 Rock hind (HQ662334) PVAVLVFCYGLICRTIWRNLKYKTRTRRTVAEANRSGMLGRSSVSSV--275
V1a1 Pupfish   (GQ981412) PVAVLVFCYGFICRAIWRNLKCKTRRKSADAVVEATKSGILGRSSVS--272
V1a1 Fugu      (AY027886) PVAALVFCYGFICRTIWKNLKCKTQRKSVEAVAEATGAGILGPCSVS--249
V2   Pupfish   (GQ981414) PAVIIAICQFRIFKEIHDNLYLKSERTIAQVKKQQQQQQTSRKNSD--261
V2   Medaka    (BAJ04637) PALIITICQIRIFREIHNNIYLKSERTVMAEVKKSDILLRFHGFKKE--295
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Figure 3.1. Amino acid sequence alignment for the third intracellular loop region of the V1a2 receptor compared with other vasotocin receptor forms in rock hind and other fish demonstrating the specificity of the antigenic site (labeled with "+") for the V1a2 receptor subtype. Genbank accession numbers are listed in parentheses.

AVT receptor IHC

Three male and two female rock hind were killed with an overdose of MS-222 (1 g l^{-1}) and a cannula was immediately introduced into the ventral aorta through which ice cold physiological saline (0.9% NaCl) with heparin (200 I.U. ml^{-1}) was administered until

the gills were pale followed by ice cold Zamboni's fixative (4% paraformaldehyde in PBS, 146 mM NaCl, 0.84 mM Na_2HPO_4, and 0.16 mM NaH_2PO_4 containing 15% saturated picric acid, pH 7.4) to fix the brain in situ. Rock hind were then decapitated and brains removed and stored overnight in Zamboni's fixative. The following day, brains were rinsed in 1X PBS and cryoprotected in 30% sucrose in 1X PBS overnight at 4°C prior to embedding in OCT and storage at -80°C. Brains were transverse sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) and stored at -80°C. Prior to use in the IHC protocol, sections were removed from -80°C and stored in a vacuum desiccator for 30 min to equilibrate to room temperature and remove condensation. Sections were then rehydrated in 1X PBS for 15 minutes then incubated in 1% hydrogen peroxide in PBS for 10 min. After washing 3X in PBS, antigen retrieval was performed by incubating the slides in a Coplin jar with citrate buffer (10mM Citric Acid, pH 6.0) in a steamer for 15 minutes. After cooling for 30 min and rinsing 3X in PBS, sections were incubated for 1 hour in blocking solution (3% normal goat serum and 0.3% TritonX-100 in PBS). Blocking solution was removed and sections were incubated with AVTr antibody (1:1000) in PBS at 4 °C overnight in a humidity chamber (Immuno Stain Moisture Chamber, Evergreen scientific, Los Angeles, CA). After incubation, sections were rinsed 3X over 45 min in PBS. Sections were then incubated for 2 hours with biotinylated goat anti-rabbit secondary antibody (1:200) (Vector Laboratories) in blocking solution, rinsed 3X in PBS and, incubated for 30 min with ABC reagent according to the manufacturer's instructions (Vector Laboratories, USA). Immunoreactivity was detected using diaminobenzidine (DAB) substrate (Impact DAB, Vector Laboratories, USA). Sections were dehydrated with an alcohol series followed by xylene and cover-slipped with Cytoseal 60 (Richard-Allan, Kalamazoo, MI).

IHC Controls

The AVTr IHC controls included: 1) omission of primary antibody, 2) omission of secondary antibody from the protocol and 3) pre-absorption of the antibody with 1 μg of blocking peptide to 1 μl antibody diluted (1:1000) overnight at 4° C prior to use in IHC experiments.

Rock hind brain atlas and photomicrographs

An atlas of the rock hind brain was generated using the most symmetrical sections from one rock hind brain. Sections were photographed on a Nikon eclipse microscope at 4X with a black and white high resolution camera (Photometrics Coolsnap cf MONO). Images were imported into Adobe Photoshop (CS-2, Adobe Systems Incorporated, San Jose CA) and a complete image of each section was created by merging 20-50 images. Outlines of sections were created using Adobe Illustrator Live Trace (CS-2, Adobe Systems Incorporated, San Jose CA). There are only a few complete cytoarchitecture studies for fish brain, and the rock hind brain morphology appeared most similar to the organization of the brain of the European seabass, *Dicentrarchus labrax*. Therefore, the terminology from the three parts series on cytoarchitectonic studies of the seabass (Cerda-Reverter et al., 2008; Cerda-Reverter et al., 2001a; Cerda-Reverter et al., 2001b) was employed as the main guide for identifying and labeling regions with supplemental information from Northcutt and Davis (1983), zebrafish brain atlas (Wullimann et al., 1996) and distribution of steroid receptors in African Cichlid (Munchrath and Hoffman, 2010).

Cells labeled by the AVTr antibody in immunohistochemistry experiments were categorized by shape and size. The presence of AVTr signal was identified by the presence of darkly stained cells from the DAB reaction and was confirmed on multiple individuals. Relative expression between regions was not quantified but representative

intensity was hand drawn as dots corresponding to three size categories. Representative photomicrographs were taken with the microscope and camera listed above and photomerged in Adobe Photoshop. Brightness and contrast were adjusted for each image for consistency. Regions of high signal were identified and labeled using the rock hind brain atlas.

During removal of the rock hind brain, the pituitary would detach and remain in the brain case. Therefore, the pituitary was processed separately and a generalized description is provided without reference to the plane of the section because proper orientation was not feasible.

RESULTS

Gross morphology

The olfactory bulbs of the rock hind are relatively small with a narrow connection to the telencephalon. The rock hind has a hypertrophied telencephalon as compared to zebrafish (Wullimann et al., 1996) and European seabass (Cerda-Reverter et al., 2001a), with five lobes visible on each hemisphere (Fig. 2). In transverse section, an extra layer adjacent to the dorsolateral part of the dorsal telencephalon (Dld) is observed and is designated Dld2. The rock hind has a large optic tectum and a large cerebellum that encroaches upon the lobes of the optic tectum (Fig. 2).

Antibody Characterization

Western blot analysis carried out using the rock hind AVT V1a2 antibody confirmed the presence of a predicted size band of ~45kDa in the membrane fraction, but not in the cytosolic or nuclear fractions (Fig. 3A). No differences were observed between male and female samples (female not shown) in Western blot. Pre-absorption of the antibody prior to application in Western blot analysis prevented the appearance of any

band in the membrane fraction (Fig. 3B). In experimental sections, DAB staining in AVTr immuno-positive cells was very dark brown compared to the background staining (Fig. 3C). Omission of primary antibody or secondary antibody (not shown) from the protocol as well as pre-absorption of the antibody with the peptide sequence (Fig. 3D) resulted in the absence of significant DAB staining indicating the specificity of the reaction.

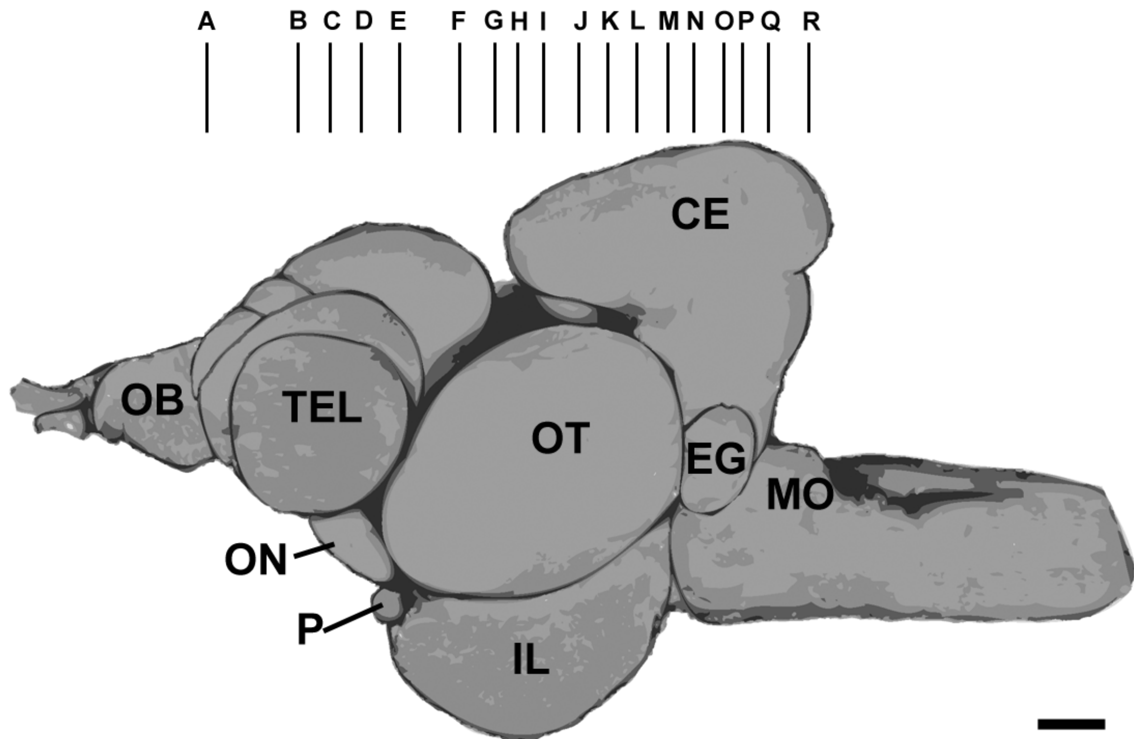


Figure 3.2. Lateral view of the rock hind *Epinphelus adsenscionis* brain. Lettered lines indicate the approximate location of the transverse sections shown in Figure 3. Abbreviations are listed in the abbreviations table. Scale bar= 1mm.

Table 3.2. Abbreviations

Abbreviation	Definition
AC	anterior commissure
AP	accessory pretectal nucleus
CCe	Corpus cerebella
CM	corpus mamillare
CP	central posterior thalamic nucleus
CZ	central zone of the optic tectum
DAO	dorsal accessory optic nucleus
Dc1	central part of the dorsal telencephalon division 1
Dc2	central part of the dorsal telencephalon division 2
Dc3	central part of the dorsal telencephalon division 3
Dc4	central part of the dorsal telencephalon division 4
Dd	dorsal part of the dorsal telencephalon
Dld1	lateral zone of the dorsal telencephalon division 1
Dld2	lateral zone of the dorsal telencephalon division 2
Dlp	posterior division, lateral part of the dorsal telencephalon
Dlv	ventral division of the lateral part of the dorsal telencephalon
Dm1	medial part of the dorsal telencephalon, subdivision 1
Dm2	medial part of the dorsal telencephalon, subdivision 2
Dm3	medial part of the dorsal telencephalon, subdivision 3
Dm4	medial part of the dorsal telencephalon, subdivision 4
DMON	decussation of the area octavolateralis
DON	descending octaval nucleus
DP	dorsal posterior thalamic nucleus
Dp	posterior part of the dorsal telencephalon
DT	dorsal tegmental nucleus
DWZ	deep white zone of the optic tectum
E	entopeduncular nucleus
ECL	external cellular layer of olfactory bulbs
EG	granular eminence
G	granular layer of the cerebellum
GL	glomerular layer of olfactory bulbs
HCo	horizontal commissure
I	intermediate thalamic nucleus
ICL	internal cellular layer of olfactory bulbs
IO	olive inferior
IP	interpeduncular nucleus
IR	inferior nucleus of the raphe
IV	fourth ventricle
LC	nucleus of the locus coeruleus
LFB	lateral forebrain bundle
LSO	lateral septal organ
LT	lateral thalamic nucleus
M	molecular layer of the cerebellum
MAG	magnocellular octaval nucleus
MLF	medial longitudinal fascicle
MON	medial octavolateral nucleus
NAPv	anterior periventricular nucleus
NAT	anterior tuberal nucleus
NC	nucleus corticalis
NCLl	central nucleus of the inferior lobe
NCW	nucleus of the commissure of Wallemberg
NDLl	diffuse nucleus of the inferior lobe
NDLlc	central part of the diffuse nucleus of the inferior lobe
NDLll	lateral part of the diffuse nucleus of the inferior lobe
NDLlm	medial part of the diffuse nucleus of the inferior lobe
NGa	anterior nucleus glomerulosus
NGp	posterior nucleus glomerulosus
NGS	secondary gustatory nucleus
NGT	tertiary gustatory nucleus
NH	habenular nucleus
NLT	lateral tuberal nucleus
NLTc	lateral tuberal nucleus, central part
NLTI	lateral tuberal nucleus, inferior part
NLTv	lateral tuberal nucleus, ventral part
NLV	lateral nucleus of the valvula
NP	paracommissural nucleus
nMLF	nucleus of the medial longitudinal fascicle
NLVa	lateral nucleus of the valvula, anterior part
NLVc	lateral nucleus of the valvula, central part
NPC	central pretectal nucleus
NPGa	anterior preglomerular nucleus
NPGc	commissural preglomerular nucleus
NPGI	lateral preglomerular nucleus
NPGm	medial preglomerular nucleus
NPOav	anteroventral part of the parvocellular preoptic nucleus
NPOpc	parvocellular part of the parvocellular preoptic nucleus
NRL	nucleus of the lateral recess
NRP	nucleus of the posterior recess
NSC	suprachiasmatic nucleus
NSV	nucleus of the saccus vasculosus
NT	nucleus taenia
nIII	nucleus of the oculamotor nerve
nIV	trochlear nerve nucleus
nVI	abducens nerve nucleus
ON	optic nerve
OT	optic tectum
PCo	posterior commissure
PE	pre-eminential nucleus
PG	periventricular granular cell mass of the caudal lobe
pgd	dorsal periglomerular nucleus
PGZ	periventricular greay zone of the optic tectum
Pj	Purkinje cells
PLl	perilemniscular nucleus, lateral part
PLm	perilemniscular nucleus, medial part
PMgc	gigantocellular part of the magnocelluar preoptic nucleus
PMmc	magnocellular part of the magnocelluar preoptic nucleus
PMpc	parvocelluar part of the magnocellular preoptic nucleus
PPd	dorsal periventricular pretectal nucleus
PPv	ventral periventricular pretectal nucleus
PS	pineal stalk
PSi	intermediate superficial pretectal nucleus
PSm	medial superficial pretectal nucleus
PT	posterior thalamic nucleus
PVO	paraventricular organ
RI	inferior reticular nucleus
RL	lateral reticular nucleus
RM	medial reticular nucleus
RS	superior reticular nucleus
S	sensory root of the facial nerve
SR	superior nucleus of the raphe
SV	saccus vasculosus
SWGZ	superficial white and gray zone of the optic tectum
T	tangential nucleus
TEG	tegmentum
TLa	nucleus of the torus lateralis
TLo	torus longitudinalis
TNgc	terminal nerve ganglion cells
TPp	periventricular nucleus of the posterior tuberculum
TS	semicircular torus
TTB	tectobulbular tract
V	trigeminal nerve
VAO	ventral accessory optic nucleus
Vc	central nucleus of the ventral telencephalon
VCe	valvula of the cerebellum
Vd	dorsal nucleus of the ventral telencephalon
Vi	intermediate nucleus of the ventral telencephalon
VIII	octave nerve
Vl	lateral nucleus of the ventral telencephalon
Vm	medial nucleus of the ventral telencephalon
VM	ventromedial thalamic nucleus
VOT	ventral optic tract
Vp	postcommissural nucleus of the ventral telencephalon
Vs	supracommissural nucleus of the ventral telencephalon
Vv	ventral nucleus of the ventral telencephalon
Xm	facial-vagal visceromotor column

Distribution of AVTr immunoreactive neuronal elements

The abbreviations used in this study are shown in Table 1. A lateral view of the rock hind brain showing gross morphological structures is presented in Figure 1 with lines indicating locations of the transverse sections illustrated in Figure 4. AVTr expression was high in the rock hind brain and was seen in all major regions including olfactory bulbs, telencephalon, preoptic area, hypothalamus, posterior tuberculum, syncencephalon, mesencephalon, and rhombencephalon. We observed AVTr expression in many different cell types and sizes: small round and fusiform cells ranging from 4 to 7 microns, medium fusiform, unipolar and bipolar cells ranging from 12 to 20 microns, and large fusiform and unipolar cells ranging from 21 to 100 microns. In the following sections we present a map for the AVT V1a receptor protein in rock hind brain drawn as qualitative expression in three categorical cell sizes as seen in IHC experiments with DAB staining. Representative photomicrographs are presented in Figure 5 that detail areas of high to moderate protein expression of AVT V1a receptor in rock hind brain sections.

Olfactory bulbs and telencephalon

In the olfactory bulbs, we observed AVTr expression in a dense population of small cells in the inner cell layer (ICL) with signal present in only few larger unipolar cells in the glomerular layer (GL). Strong immunoreaction was seen in large terminal nerve ganglion cells (TNGcs) located at the medial margin of the olfactory bulbs (Figs. 4A, 5A-B).

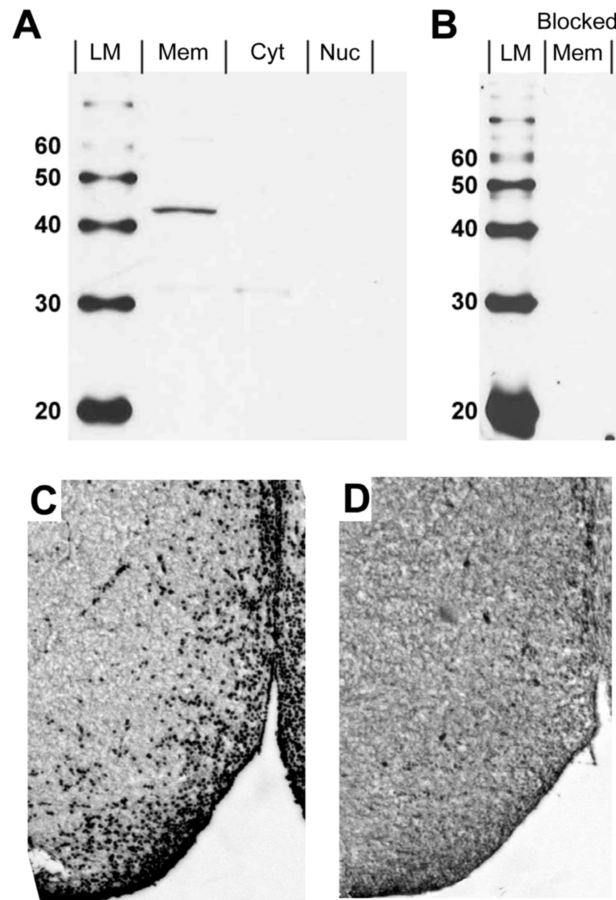


Figure 3.3. (**A**) Western blot of rock hind brain tissue compartments labeled with the AVT V1a receptor antibody showing a prominent band of the predicted size (45 kDa) as estimated with a lane marker (LM) in the membrane fraction (Mem) but not in the cytosolic (Cyt) or nuclear (Nuc) fraction. (**B**) Western blot of rock hind brain membrane fraction incubated with the AVT V1a receptor antibody blocked with antigenic peptide showing the absence of any band. Transverse sections of rock hind ventral telencephalon with (**C**) showing cells labeled by the AVT V1a2 antibody (1:1000) and secondary HRP antibody (1:200), visualized with DAB and (**D**) AVTr antibody pre-absorbed with 1μg.l^{-1} synthetic peptide that the antibody was raised against showing the absence of specific signal.

In the ventral telencephalon, there was a very dense expression of AVTr in small round cells in the lateral septal organ (LSO; Figs. 4B, 5D) and entopeduncular nucleus (E; Figs. 4D, 5F). The dorsal nucleus of the ventral telencephalon (Vd; Figs. 4B-C, 5D),

intermediate nucleus (Vi; Figs. 4D-E, 5F), and ventral nucleus (Vv; Figs. 4D-E, 5D-E) had many small round cells that were AVTr-postitive with the highest expression occurring along the ventricular wall. The central nucleus of the ventral telencephalon (Vc) had dense AVTr-positive cells in a cluster (Figs. 4B-C, 5B). All other regions in the ventral telencephalon, the supracommissural nucleus of the ventral telencephalon (Vs; Figs. 4B-E, 5D), the postcommissural nucleus of the ventral telencephalon (Vp; Fig. 4D), and the lateral nucleus of the ventral telencephalon (Vl; Figs. 4B-C, 5D) had many scattered small cells that were AVTr-positive.

We identified four medial divisions of the dorsal part of the telencephalon as Dm1-4. All Dm regions had AVTr expression in small round cells, with the highest expression in subdivision 1 (Dm1; not shown) and subdivision 2 (Dm2; Figs. 4A-C). The dorsolateral part of the dorsal telencephalon had two distinct regions: the outer margin, division 1 (Dld1) was characterized by many small round cells expressing AVTr (Figs. 4B-D, 5C) and a previously undescribed, inner division 2 (Dld2) had many medium sized AVTr-positive cells (Fig. 4B-D, 5C). All divisions of the lateral part of the dorsal telencephalon: posterior (Dlp; Fig. 4D), ventral division (Dlv; Figs. 4B-D) and lateral part of the dorsal telencephalon (Dd; Figs. 4B-C, 5C) had many small round cells expressing AVTr. We observed the highest expression in this area in small round cells in the nucleus taenia (NT; Figs. 4B-D, 5D).

In the four major divisions of the central part of the dorsal telencephalon identified in rock hind brain, Dc1 had few small round cells expressing AVTr (Figs. 4A-C, 5C), whereas Dc2 and Dc3 had many medium sized, bipolar cells (Figs. 4B-D, 5C) and Dc4 had only few medium bipolar cells (Figs. 4C-D, 5C) that were AVTr-positive. In the posterior part of the dorsal telencephalon (Dp) AVTr expression was observed in only small round cells (Fig. 4E).

Preoptic area and hypothalamus

In the preoptic area, we observed densely packed small AVTr-expressing cells in the anteroventral part of the parvocellular preoptic nucleus (NPOav) arranged tightly around the preoptic recess (Figs. 4C-E, 5E-F). The parvocellular part of the parvocellular preoptic nucleus (NPOpc) had many small AVTr-expressing cells packed against the third ventricle and fewer small and medium cells scattered laterally (Figs. 4C-E, 5E-F). The parvocellular part of the magnocellular nucleus (PMpc) had more densely packed small cells expressing AVTr arranged in rows near the third ventricle (Figs. 4D, 5F). The magnocellular part of the magnocellular preoptic nucleus (PMmc) had many AVTr-positive, medium sized cells along the margin of the third ventricle (Figs. 4D, 5F). The gigantocellular part of the magnocelluar preoptic nucleus (PMgc) had very few large cells that expressed AVTr (Figs. 4D, 5F-G). The anterior periventricular nucleus (NAPv) had many AVTr-positive, small cells around the margin of the third ventricle and few small cells scattered laterally (Figs. 4F, 5G).

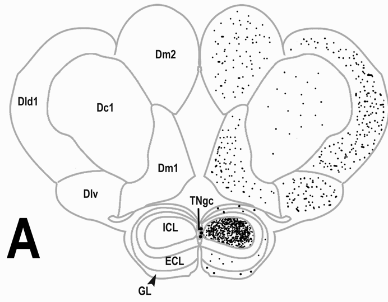
In the ventral thalamus, the ventromedial thalamic nucleus (VM) had many small round cells expressing AVTr at the margin of the third ventricle and larger fusiform cells scattered laterally (Figs. 4F-G, 5G). The intermediate thalamic nucleus (I) had dense, small AVTr-expressing cells projecting ventrally from the habenular nucleus (NH) (Figs. 4F, 5G). In the dorsal thalamus, we observed expression in the accessory pretectal nucleus (AP) with many small AVTr-positive cells in a ventromedial orientation (Fig. 4G). The dorsal posterior thalamic nucleus (DP) and the central posterior thalamic nucleus (CP) both had many small AVTr-positive cells (Figs. 4G-H, 5I).

In the epithalmaus**,** the habenular nucleus (NH) was packed with small cells that were AVTr-positive (Figs. 4F, 5G). Although we were unable to preserve the entire

pineal organ structure, ventral projections at the base of the pineal stalk (PS) had small and medium AVTr-positive cells (Fig. 5G).

In the hypothalamus, we observed AVTr expression in very densely packed small cells in the nucleus of the lateral recess (NRL), especially around the margin of the recess (Fig. 4H-N, 5K, M, R). In several parts of the lateral tuberal nucleus: lateral, ventral and dorsal (NLTl, NLTv and NLTd, respectively) we observed small cells densely packed around the margin of the third ventricle and ventral recess that were AVTr-positive (Figs. 4F-G, 5H-I). Other posterior portions of NLT had only scattered expression in small cells (Figs. 4H-I, 5H). The nucleus of the posterior recess (NRP) had a very dense population of small, round AVTr-positive cells (Figs. 4I-K, 5K, M). Both the nucleus of the saccus vasculosus (NSV; Figs. 4K) and the saccus vasculosus (SV) were both heavily stained for AVTr (Figs. 4L-P, 5M), where medium sized laminar cells lined the inner recess of the NSV and the outer folds of the SV.

We observed AVTr expression in several cell types within the inferior lobe of the hypothalamus. The central nucleus of the inferior lobe (NCLI; Figs. 4M, 5R) had expression in many medium sized cells, whereas the lateral part of the diffuse nucleus of the inferior lobe (NDLIl; Figs. 4H-M, 5R) and the medial part of the diffuse nucleus of the inferior lobe (NDLIm; Figs. 4M, 5R) had expression in many small round and fusiform cells.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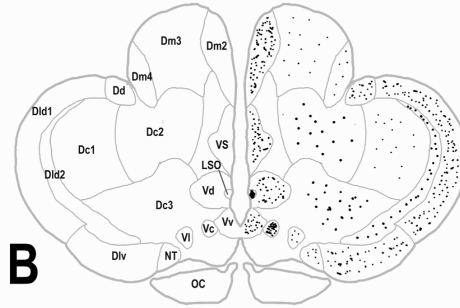
Dc1
Dm1
Dlv
TNgc
ICL
ECL
GL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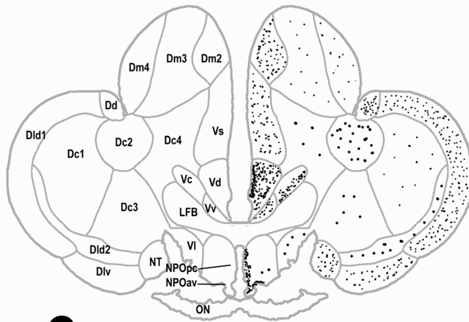
NT
OC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Dd
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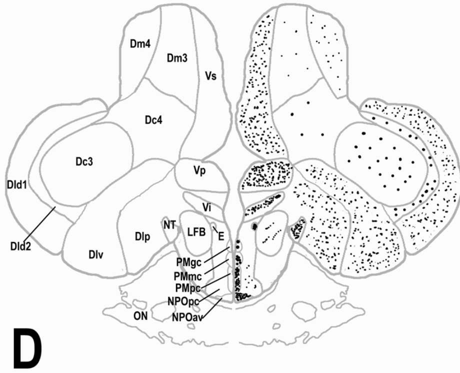
Vl
Dld2
NT
NPOpc
Dlv
NPOav
ON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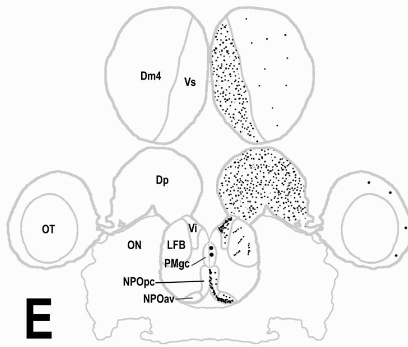
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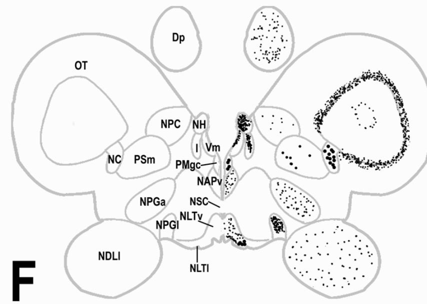
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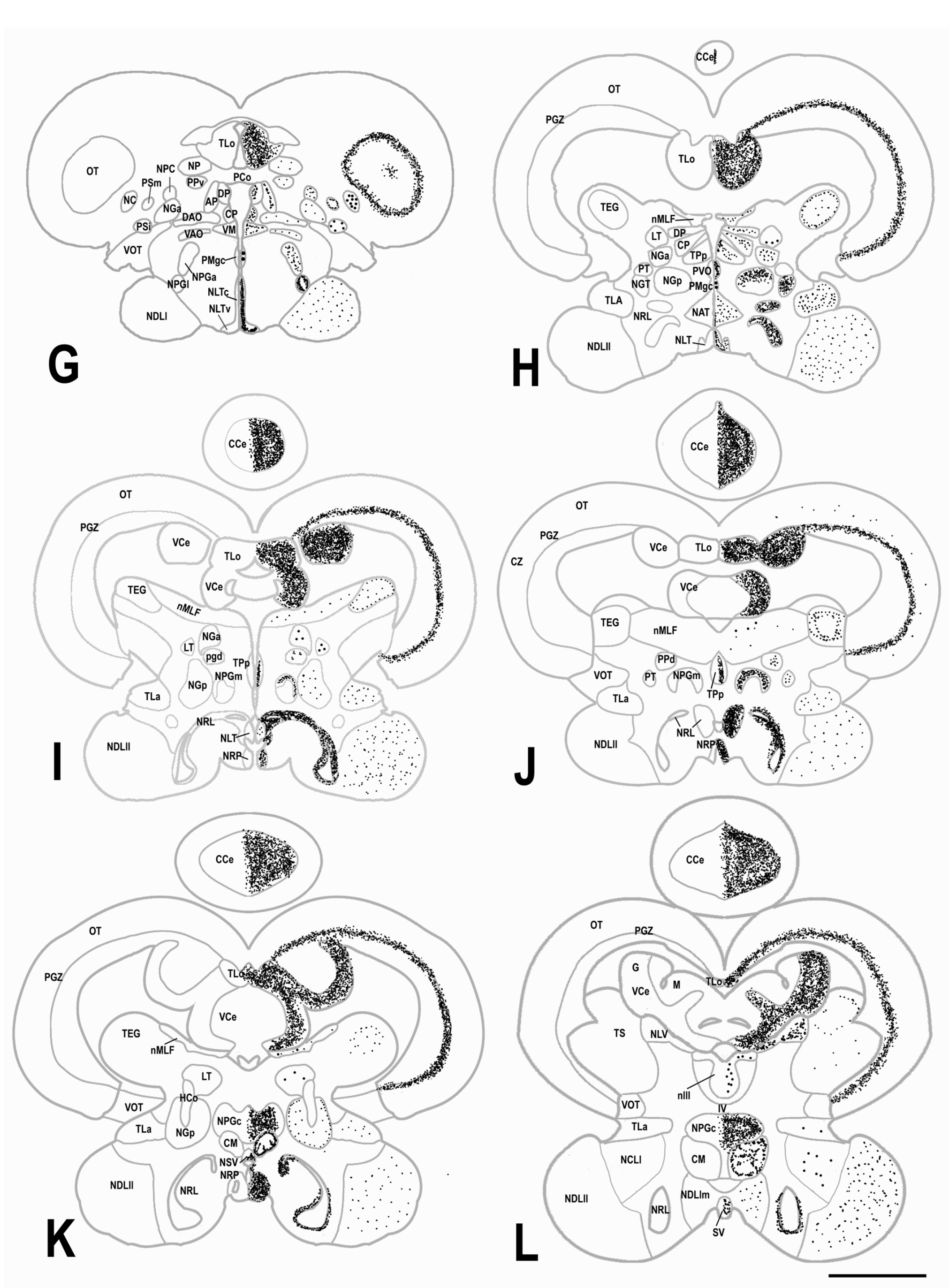

G
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AP
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DAO
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VAO
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TPp
PT
PVO
NGT
NGp
PMgc
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NLT
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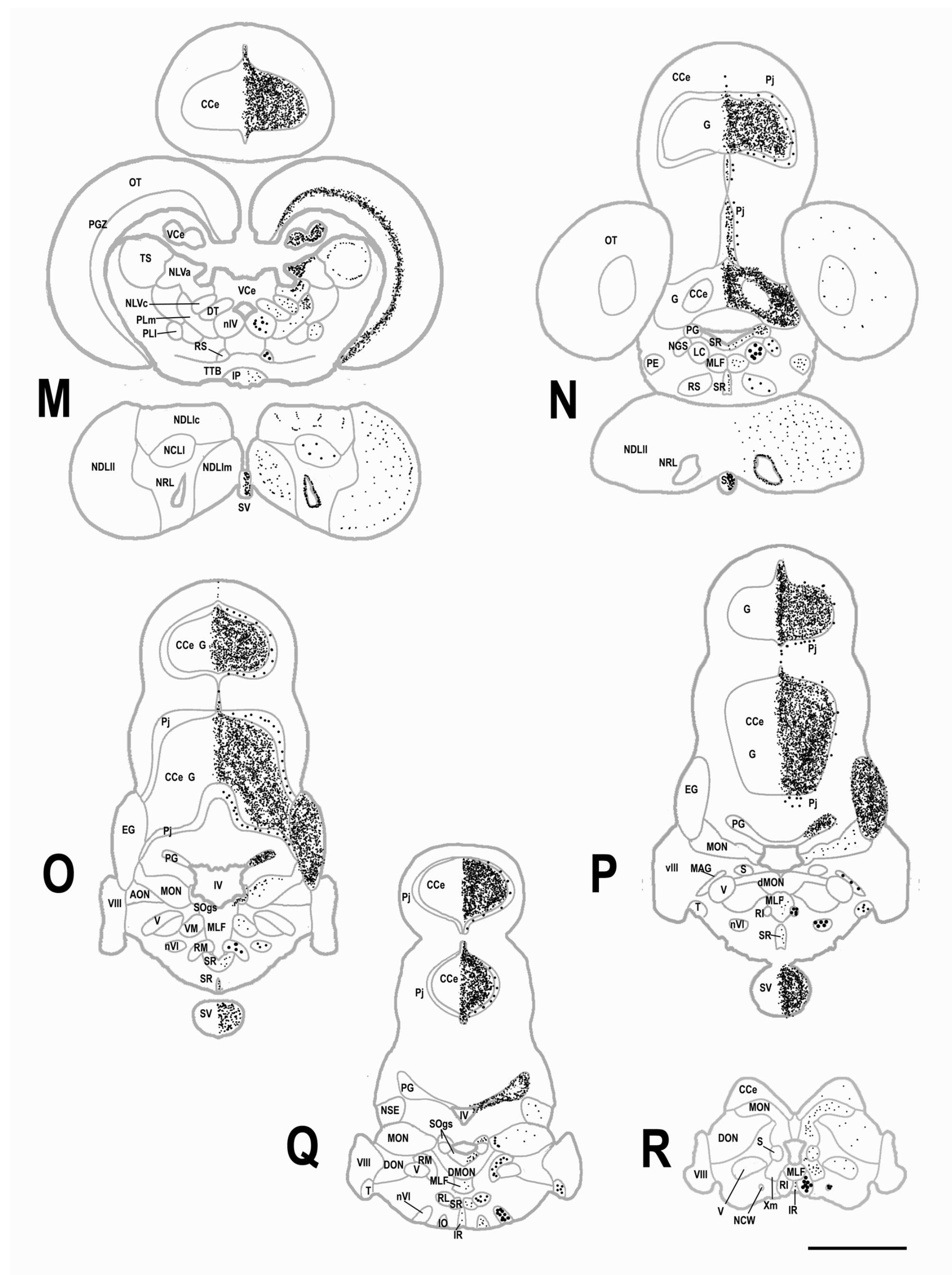


Figure 3.4. A-R: Diagrammatic representation of AVT V1a receptor expression by the brain region, and relative cell size (small, medium and large dots) and signal intensity as seen in 20μm transverse sections of rock hind brain passing through the regions indicated in Figure 1. For abbreviations, see Table 2. Scale bar = 2mm.

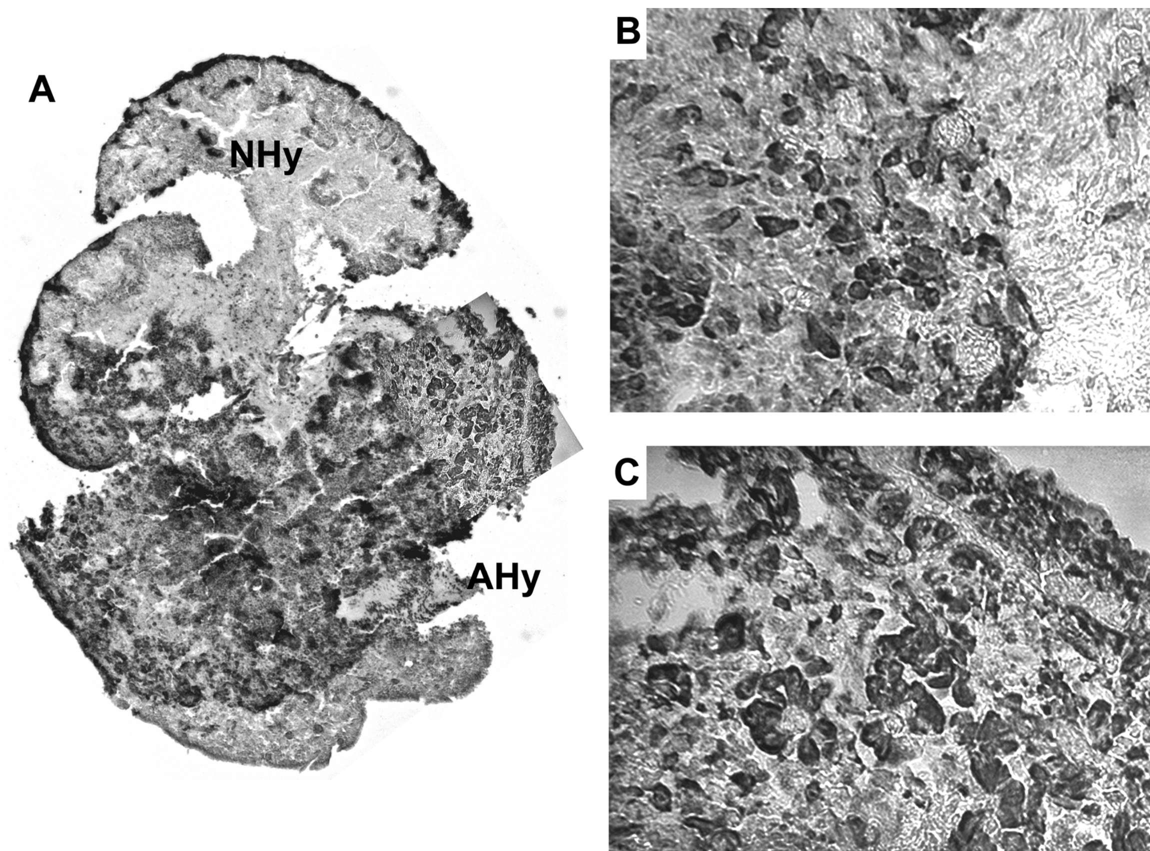


Figure3.6: Photomicrographs of rock hind pituitary expressing AVT V1a receptor visualized by immunohistochemistry using AVTr (1:1000) and secondary HRP (1:200) antibodies and labeled with DAB. **A.** AVTr expression in the adenohypophysis but not the neurohypophysis. **B-C.** Higher magnification of **A** showing AVTr expression in cellular structures and formations. For abbreviations see Table 2. Scale bars = 200μm.

Pituitary gland

In the pituitary (PIT), AVTr expression was observed in the adenohypophysis (AHy) but not in the neurohypophysis (NHy; Fig. 6A). Within the AHy, different medium and large cell types and cell cluster configurations were AVTr-positive. Higher magnification images of representative cell types are presented in Figures 6B-C.

Posterior tuberculum and syncencephalon

We observed high expression of AVTr in the posterior tuberculum. The periventricular nucleus of the posterior tuberculum (TPp; Figs. 4I-J, 5I) and the paraventricular organ (PVO; Figs. 4H, 5H-I) had AVTr expression in densely packed small cells particularly, near the ventricular wall. AVTr expression was observed in small cells in all regions of the preglomerular nucleus: many scattered cells in the anterior preglomerular nucleus (NPGa; Fig. 4G); lateral preglomerular nucleus (NPGl; Fig. 4G); densely packed cells dorsally in the medial preglomerular nucleus (NPGm; Figs. 4I-J, 5K); and a large area of small, dense AVTr-expressing cells in the commissural preglomerular nucleus (NPGc; Figs. 4K-L, 5M). The anterior nucleus glomerulosus (NGa; Figs. 4G-J) had many scattered medium fusiform shaped cells expressing AVTr and the posterior nucleus glomerulosus (NGp; Figs. 4H-K, 5I) had many small cells expressing AVTr surrounding larger cells that had no signal. The tertiary gustatory nucleus (NGT) had many small cells expressing AVTr (Fig. 4H). The corpus mamillare (CM) had very dense AVTr-positive cells in clusters around the margin of the lobes (Figs. 4K-L, 5M). The torus lateralis (TLa; Figs. 4H-L) had many small and few medium cells expressing AVTr, while the lateral thalamic nucleus (LT; Figs. 4I, K, 5I) had few scattered medium sized cells that were AVTr-positive.

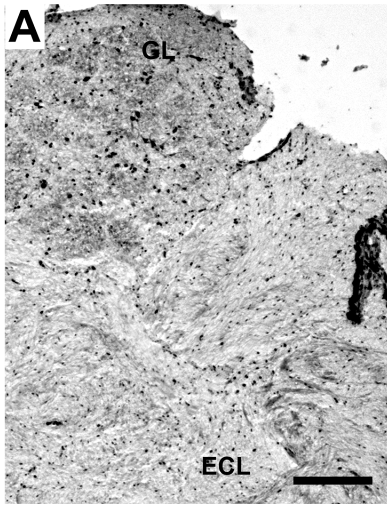
In the synencephalon (the region between the diencephalon and the mesencephalon as described by Braford and Northcutt (1983), the dorsal periventricular pretectal nucleus (PPd) (Fig. 4J) had many small cells that were AVTr-positive. The nucleus of the medial longitudinal fasciculus (nMLF; Figs. 4H-J, N-R, 5L, S-U) had few scattered small and medium cells that were also immuno-positive for AVTr.

In the pretectum, we observed the highest AVTr expression in densely packed medium sized cells in the nucleus corticalis (NC; Figs. 4F-G, 5J). The central pretectal

nucleus (NPC; Figs. 4F-G, 5J) and the magnocellular superficial pretectal nucleus (PSm; Figs. 4F-G, 5J) had only scattered small cells expressing AVTr. The intermediate superficial pretectal nucleus (Psi; Figs. 4G, 5J) had medium cells expressing AVTr around the margin of the round glomerular neuropil. The accessory pretectal nucleus (AP) (Fig. 2G) had a narrow margin of small dense cells expressing AVTr adjacent to DP and CP. We observed only few scattered fusiform and round cells in the dorsal and ventral accessory optic nuclei (DAO, VAO; Fig. 4G).

Mesencephalon

In the optic tectum (Figs. 4F-N) we identified four distinct zones pictured in Fig. 5L. Very little AVTr expression was seen in the central zone of the optic tectum (CZ) and interspersed in nerve fibers of the deep white zone of the optic tectum (DWZ). We observed the highest AVTr expression in small dense cells within the periventricular gray zone (PGZ). In the anterior portion of the optic tectum, we also observed small cells lining the periventricular region that were AVTr-positive (Fig. 4F). No AVTr expression was seen in the superficial white and gray zone. Similar to the PGZ, we observed high expression in the torus longitudinalis (TLo) consisting of small dense cells (Figs. 4G-L, 5L).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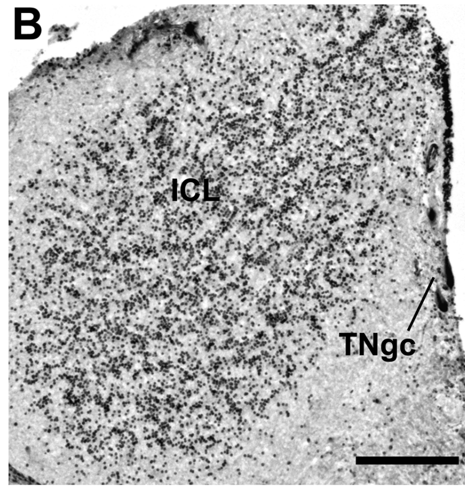
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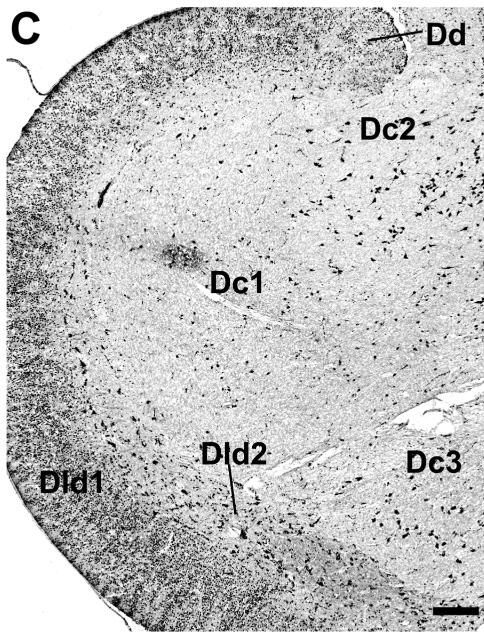
TNgc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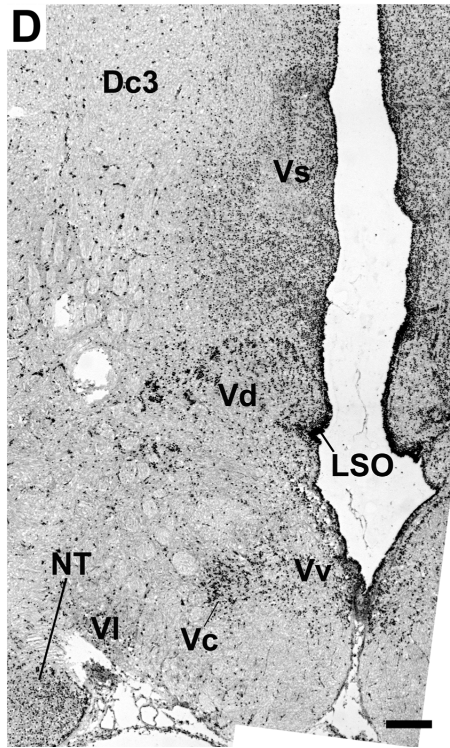
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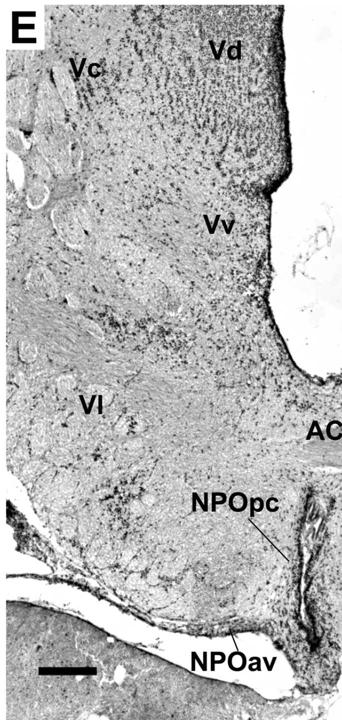
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Vd
Vv
Vl
AC
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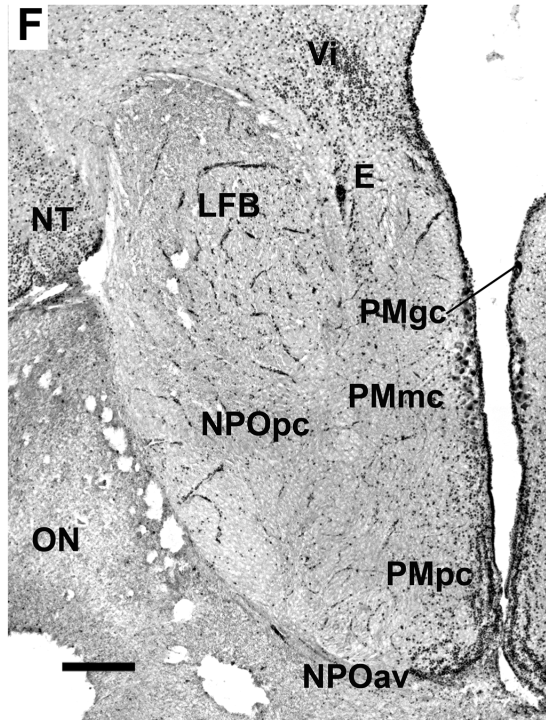
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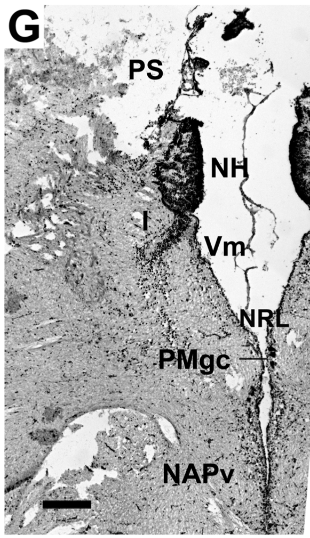
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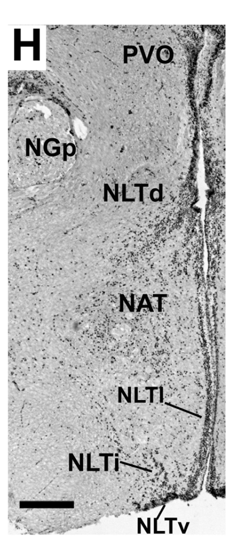
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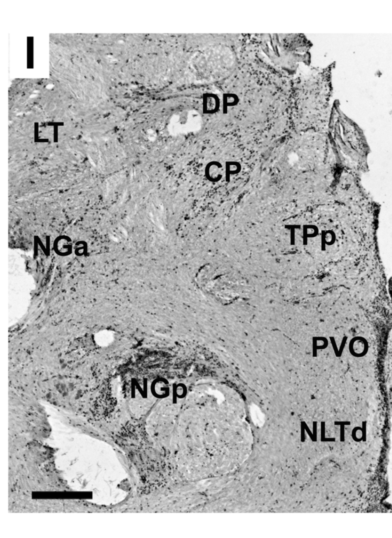
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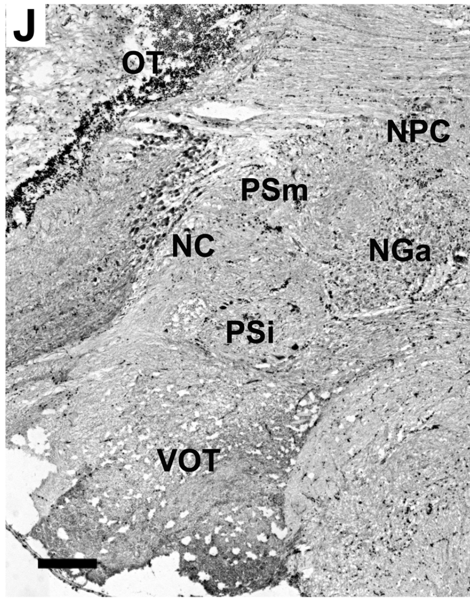
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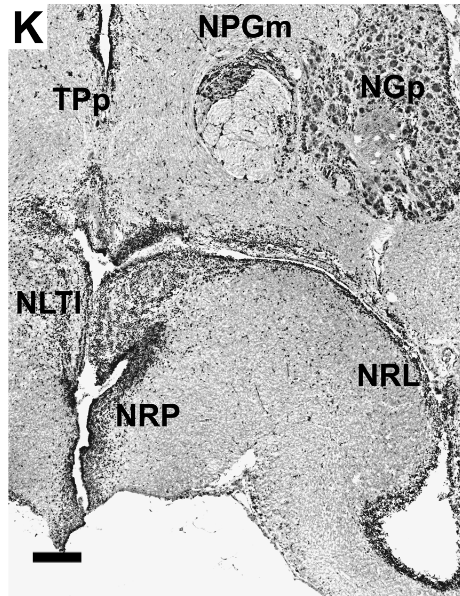
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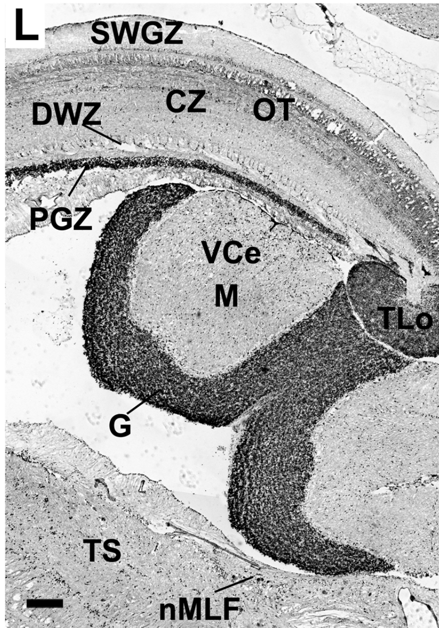
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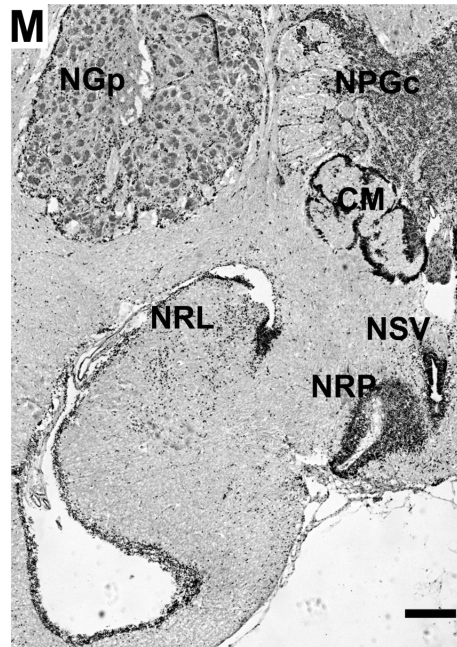
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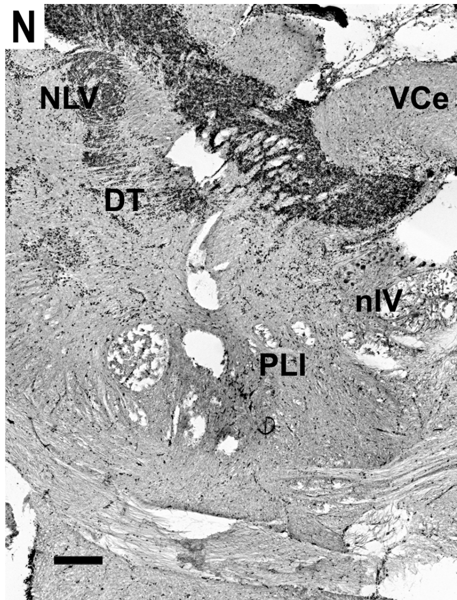
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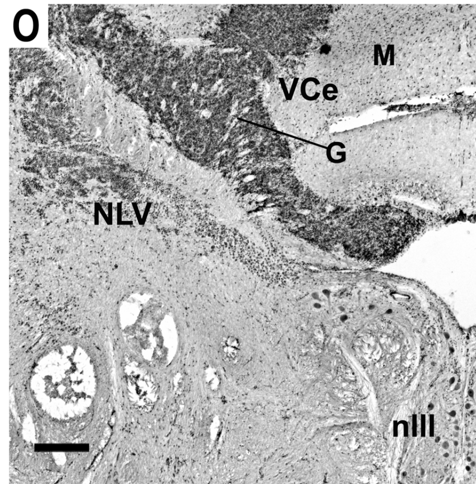
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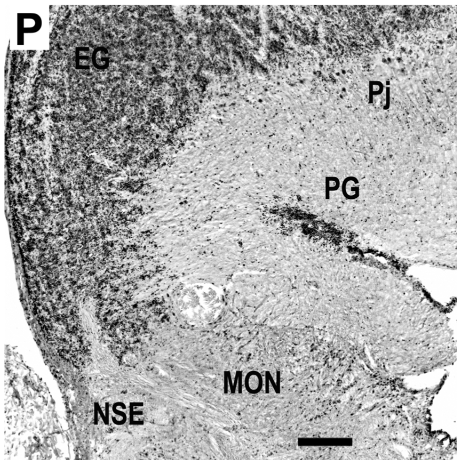
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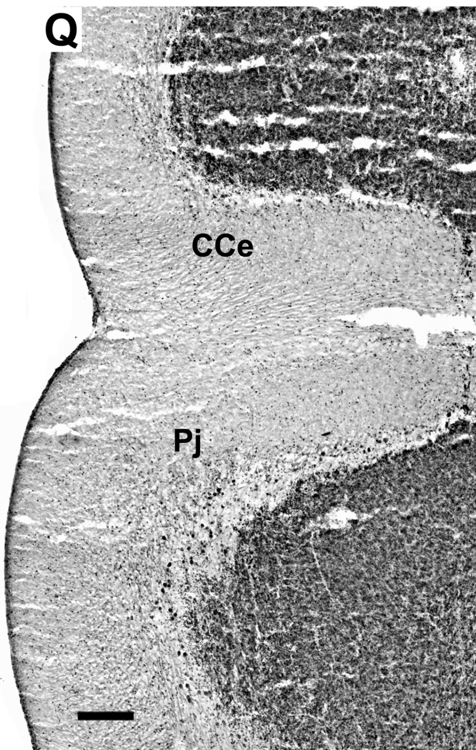
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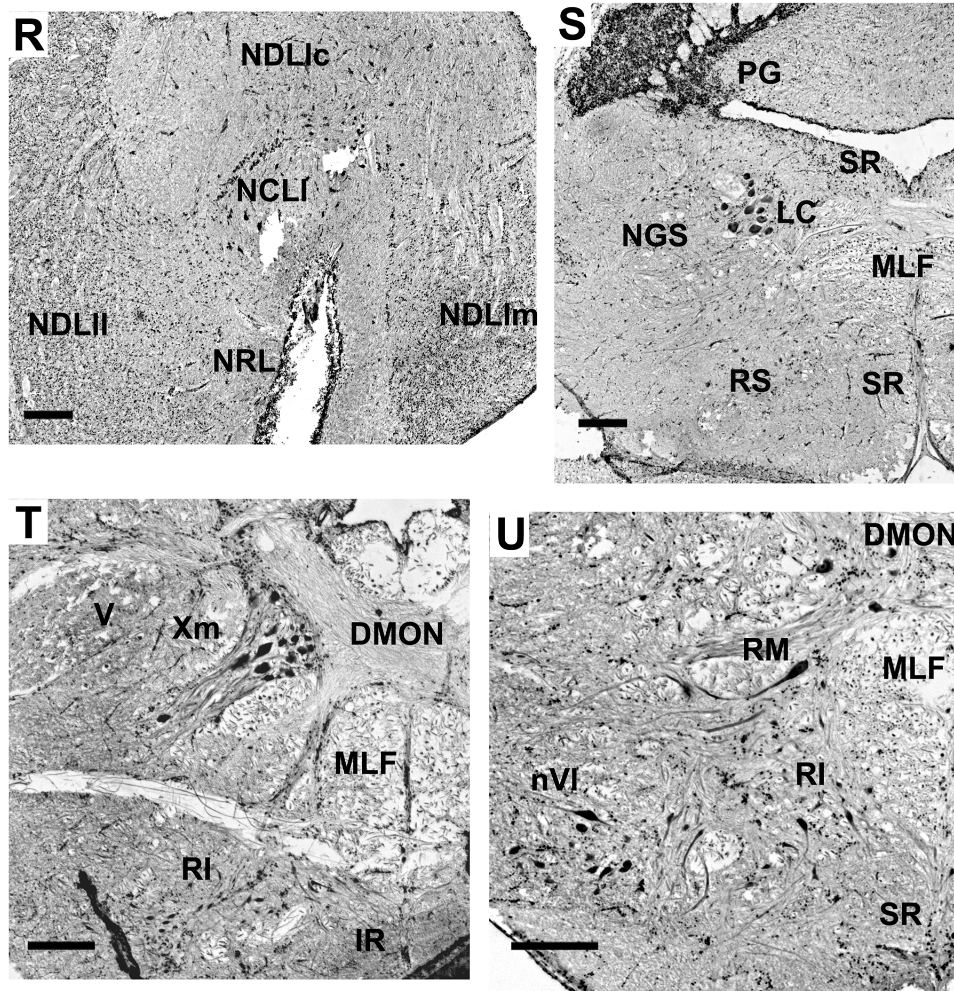

Figure 3.5. Photomicrographs of rock hind brain sections showing AVT V1a receptor expression in numerous regions and cell types visualized by immunohistochemistry using AVTr (1:1000) and secondary HRP (1:200) antibodies and labeled with DAB. **A-B**: olfactory bulb; **C-D**: telencephalon; **E-F**: preoptic area nuclei; **G**: pineal stalk (PS) and habenular nuclei (NH); **H-I**: lateral tuberal nuclei; **J**: pretectum; **K**: nuclei of lateral and posterior recesses; **L**: zones of optic tectum, valvula of the cerebellum (VCe) and torus semicircularis (TS); **M**: corpus mamillare (CM) and commissural preglomerular nucleus (NPGc); **N**: VCe, lateral nucleus of the valvula (NLV) and dorsal tegmental nucleus (DT); **O**: granular (G) and molecular (M) layers of VCe, and nucleus of the oculomotor nerve (nIII); **P**: granular eminence (EG) and periventricular granular cell mass of the caudal lobe (PG); **Q**: corpus cerebellum (CCe) and Perkunje cell layer (Pj); **RB:** regions of the inferior lobe; **S**: PG and nucleus of the locus coeruleus (LC); **T**: facial-vagal visceromotor column (Xm) and inferior reticular nucleus (RI); **U**: medial reticular nucleus (RM), decussation of the area octavolateralis (DMON) and abducens nerve nucleus (nVI). For additional abbreviations see Table 2. Scale bars = 200µm.

Rhombencephalon

In the valvula of the cerebellum (VCe; Figs. 4G-M, 5L, N-O) and the corpus cerebellum (CCe; Figs. 4H-R, 5Q), we observed densely packed granular cells expressing AVTr. Purkinje cells (Pj) within the CCe (Figs. 4N-Q, 5Q) also were AVTr-positive. Within the vestibulolateral lobe, the granular eminence (EG; Figs. 4O-P. 5P) and the periventricular granular cell mass of the caudal lobe (PG; Figs. 4N-Q, 5P) had densely packed small cells expressing AVTr.

In the reticular formation, the superior raphe nucleus (SR) had many small cells expressing AVTr (Figs. 4N-Q, 5S,U) and inferior raphe nucleus (IR) had fewer small and medium cells expressing AVTr (Figs. 4Q-R, 5T). The superior reticular nucleus (RS) had few medium and large cells expressing AVTr (Figs. 4M-N, 5S). The medial reticular nucleus (RM; Figs. 4O-Q, 5U) and caudally, the inferior reticular nucleus (RI; Fig. 4P-R, 5T-U) had medium and large cells expressing AVTr, whereas the lateral reticular nucleus (RL) was only populated by medium cells that were AVTr-positive (Figs. 4Q, 5T).

In the area octavolateralis, the magnocellular octaval nucleus (MAG) had a thin lamina of large fusiform cells expressing AVTr (Fig. 4P). In the tangential nucleus (T) we observed a small cluster of medium sized cells that were AVTr-positive (Figs. 4P-Q). The medial octavolateral nucleus (MON) had only few small and medium cells expressing AVTr (Figs. 4O-R, 5P), and the descending octaval nucleus (DON) had few scattered small cells expressing AVTr (Figs. 4Q-R).

In the rhombencephalic somatomotor nuclei, we observed many large cells expressing AVTr in the abducens nucleus (nVl; Figs. 4O-Q, 5U). Within the rhombencephalic visceromotor nuclei, the trigeminal motor nucleus (VIIIm) also had large cells expressing AVTr. We were unable to differentiate the facial, vagal, and, glossopharyngeal nucleus; therefore, the collective designation facial-vagal visceromotor

column (Xm) was used as in Reverter et al. (2008). The highest expression of AVTr in this brain region was seen in large motoneurons within nerve bundles of the facial-vagal visceromotor column (Xm; Figs. 4R, 5T).

We observed many small cells that were AVTr-positive in the nucleus of the Locus coeruleus (LC; Figs. 4N, 5S), pre-eminential nucleus (PE; Fig. 4N) and interpeduncular nucleus (IP; Fig 4M). The secondary gustatory nucleus (NGS) had few medium cells expressing AVTr (Figs. 4N, 5S) and the inferior olive (IO) had a group of small cells arranged dorso-medially that were AVTr-positive (Fig. 4Q).

No obvious morphological sex differences were noted in the expression of AVTr in any region of the rock hind brain. However, these fish were captured outside of the reproductive season and therefore differences between the sexes in reproductively mature fish and/ or quantitative differences might exist that were not investigated in this study.

DISCUSSION

The present study describes for the first time distribution of the AVT V1a receptor protein expression throughout the brain of a fish species. The specificity of the antibody used in this study has been confirmed by identification of a band of expected size in Western blots as well as pre-absorption with the peptide against which the antibody was raised.

The finding of wide distribution of AVTr protein expression in a number of regions throughout the brain of rock hind is consistent with mRNA distributions of brain microdissections in this (Chapter 4), as well as in other fish and amphibian species (Hasunuma et al., 2007; Lema, 2010) and in rat (Ostrowski et al., 1994). AVTr was also localized in several areas associated with external sensory processing such as olfaction,

vision, lateral line function, and image processing (Wullimann, 1998). The cell types and expression pattern of the AVT V1a receptor in this study indicate that AVT may act as a neuromodulator via this V1a receptor subtype. AVTr expression in this study was observed in cell bodies but not in small fibers throughout the brain. This implies that the site of action for AVT for this receptor is on the membranes of cell bodies.

Although there are few published studies in fish to compare with the present V1a receptor distribution in rock hind, the distribution of the AVT V1a receptor reported here followed that reported for brain regions in autoradiography experiments with seabass (Moons et al., 1989). Similar expression patterns were seen in this study in the hind brain and reticular formation nuclei as has been reported previously in a study using fluorescently labeled AVT in newt (Lewis et al., 2005). Although definitive homology is difficult to determine in many regions of the brain while comparing AVP V1a mapping in the rhesus monkey to the AVT V1a distribution observed in this study, several regions that are clearly homologous such as the corpus mamillare (CM), the inferior olive (IO), and locus coeruleus (LC) express AVTr in both species, potentially indicating a conservation of similar functions throughout a wide range of vertebrates.

In rock hind brain, AVTr was expressed in regions associated with behavior and reproduction in other vertebrates such as the preoptic area nuclei (Foran and Bass, 1999; Liu et al., 1997), the tuberal nuclei (Wullimann, 1998) and terminal nerve ganglion cells (TNgc) (Yamamoto et al., 1997). Furthermore, AVTr was expressed in the cell populations within the pPOAH, mPOAH and gPOAH where cells producing AVT and GnRH have been localized in other species (Godwin et al., 2000; Gothilf et al., 1996; Maruska et al., 2007; Mohamed et al., 2005; Senthilkumaran et al., 1999). Although there are no published data on specific cell populations that co-express AVT and GnRH, mRNA expression studies have revealed high expression of both in the POAH of rock

hind brain (R. Kline, unpublished data). With AVTr expression in this region there is a potential for feedback on neuropeptide hormone producing neurons such as GnRH and AVT via AVTr within the POAH. Interestingly, high expression of AVTr was observed also in the thalamic nuclei of rock hind brain, the region that has been linked to sexually dimorphic, temporary color patterning and release of gametes in a simultaneous hermaphrodite (Demski and Dulka, 1986; Gothilf et al., 1996).

In rock hind lateral pallium, AVTr expression was observed in Dld1 and a previously undescribed region designated Dld2. In goldfish, lesions of the lateral pallium abolish geometric spatial learning in controlled experiments (Vargas et al., 2006). Both male and female rock hind are very territorial and make use of even small visual landmarks to designate territories (R. Kline unpublished data). AVT action in these regions of the lateral pallium could modulate spatial recognition in the males of this very territorial species and should be investigated.

AVTr was highly expressed in the habenular nuclei (NH) and pineal stalk in rock hind. Given the evidence for an AVT melatonin interaction (Kulczykowska, 1995) and further evidence in flounder that infusion of AVT causes a decrease in circulating melatonin (Kulczykowska et al., 2001), it is possible that AVT acts via the V1a receptor reported here, potentially as a modulator of melatonin production as suggested in rat (Stehle et al., 1991).

Pituitary expression of the AVT v1a receptor reported in this study was surprisingly high given that, in other taxa, the effects of AVT in the pituitary are typically attributed to a V1b receptor subtype (Balment et al., 2006; Birnbaumer, 2000; Jurkevich et al., 2005). In the rock hind pituitary, expression was limited to the adenohypophysis and not the neurohypophysis, similar to that reported in the autoradiography study of seabass (Moons et al., 1989). However, the mRNA tissue distribution studies in mammals

and amphibians reveal a considerable overlap in the expression of V1a and V1b sub-types in the pituitary (Hasunuma et al., 2007; Orcel et al., 2002).

Although the cell types documented here were not characterized, the effects of AVT have been noted on gonadotropin release in Poecilia latipinna (Groves and Batten, 1986) and AVP V1a receptors have been co-localized with FSH and LH producing cells in rat (Orcel et al., 2002). In chicken, the V1b receptor was expressed on ACTH - producing cells (Jurkevich et al., 2005); curiously, some cell types and formations appear similar to those reported here for rock hind V1a2 receptor expression. In newt and pupfish, several AVT receptor subtypes are expressed in the pituitary (Hasunuma et al., 2007; Lema, 2010). The high expression of AVT V1a2 receptor in this study in several pituitary cell types could be investigated by co-localization with corticotrophin and gonadotropin producing cells to infer function and warrants further investigation.

Many regions identified for monamine synthesis had high expression for AVTr such as the LC, a noradrenaline producing region; serotonin producing regions: the raphe nuclei, NRL NRP, NPPv (Kah and Chambolle, 1983; Khan and Thomas, 1993) and PVO (Fryer et al., 1985); and dopaminergic regions: the NLTa, RPO and NPP (Rink and Wullimann, 2002). Thus, AVTr could potentially mediate AVT actions on these monoamine neurotransmitter systems.

AVTr was highly expressed in rhombencephalon of rock hind, specifically in the reticular formation and motor control centers that have been linked to the control of sexual behavior such as clasping and response to pheromones in newt (Lewis et al., 2005 and references therein). Hindbrain AVTr expression has also been implicated in the control of vocalization in bullfrog, Rana catesbeiana (Boyd, 1997). In addition, social approach in goldfish is linked to these hind brain areas (Walton et al., 2010) that had high AVT V1a receptor expression in rock hind in this study.

The highest expression and density of AVTr that we observed in this study was in small round densely packed granular formations in the inner cell layer (ICL) of the olfactory bulb, torus longitudinalis (TLo), granular cerebellum(G), valvula cerebella (VCe), nuclei of the lateral and posterior recess (NRL and NRP), and granular eminence (EG). Although these regions appeared similar in cell type and formation, there is no apparent linkage in function between these areas to date.

The administration of AVT in fish and amphibians to alter behavior has differing effects in fish with differing reproductive strategies. AVT has been shown to increase courtship behavior and aggression in the bluehead wrasse and white perch (Salek et al., 2002; Semsar et al., 2001), and to decrease male specific behavior in damselfish and pupfish (Lema and Nevitt, 2004; Santangelo and Bass, 2006). In the newt, AVT injections increase male responses to female visual and olfactory stimuli (Thompson and Moore, 2000). Intracerebroventricular injections of AVT have been shown to be more effective at much lower doses in eliciting a response in fish and amphibians (Boyd, 1991; Salek et al., 2002; Santangelo and Bass, 2006) and so it is possible that behavioral effects of AVT could be mediated through a receptor homologous to the V1a receptor reported in this study.

Considering the wide distribution of the AVT V1a receptor throughout the brain of rock hind and potentially in other species, behavioral data using large doses of AVP V1a receptor antagonists such as Manning compound should be treated with caution. The antagonists could affect many different pathways within the brain causing the observed behavioral responses.

CONCLUSIONS

This is the first comprehensive localization study on the AVT V1a receptor in a teleost fish. We observed a wide distribution of the receptor throughout the brain areas similar to that seen in a study of rat AVP V1a mRNA distribution (Ostrowski et al., 1994). Distinctive regions associated with a wide range functions were identified such as behavior, reproduction and spatial learning, as well as sensory functions such as vision, olfaction and lateral line sensory processing. Functional and co-localization studies are needed to verify the specific relationship of the V1a receptor to particular cellular functions.

Chapter 4: Molecular Characterization and Expression of the Arginine Vasotocin System and GnRH in Relation to Behavioral Sex Change in the grouper, *Epinephelus adscensionis*

ABSTRACT

Although a potential linkage between the vasotocin/ vasopressin and gonadotropin releasing hormone systems has been proposed in several vertebrate classes, a pathway connecting behavior and reproduction has not been conclusively demonstrated. The major objective of this research was to test the hypothesis that AVT could act on the GnRH system via a vasotocin V1a receptor to influence sexual behavior and reproductive development in a sex changing grouper species. AVT, GnRH and two AVT receptor cDNAs were isolated and characterized for rock hind and calibrated assays were developed to measure levels of mRNA expressions using quantitative real time PCR (qRT-PCR). Translation of potential V1a-type cDNA sequences revealed that two distinct forms of the AVT V1a receptor occur in rock hind brain similar to that recently reported for pupfish. Immunohistochemistry (IHC) experiments co-localized GnRH with the AVT V1a2 receptor identifying a linkage for the first time between the AVT system and GnRH. The results of the IHC experiments revealed for the first time that GnRH neurons in the preoptic area express V1a2 receptors, implicating a pathway for AVT to act on GnRH. Furthermore, significantly higher levels of the AVT V1a2 receptor and GnRH transcripts were seen in male as compared to female rock hind providing further evidence of a potential action. In male rock hind, but not females, a negative relationship was seen between the V1a1 receptor and plasma 11-KT levels while a positive trend was seen the V1a2 receptor, indicating that these receptor forms differ in their regulation in males. Time series experiments using dominant male removal as a stimulus for sex change in the largest remaining fish proved inconclusive in regards to the AVT and

GnRH system while increased levels of 11-KT along with high levels of E2 indicated that sex change was not complete. However, large females undergoing sex change sampled at 10d after male removal had significantly higher levels of AVT expression compared to medium and small fish implicating a function for AVT during the development of social hierarchy.

INTRODUCTION

The neuropeptide hormones arginine vasotocin (AVT) and gonadotropin releasing hormone (GnRH) are two hormones that co-occur in the preoptic area and anterior hypothalamus (POAH), an important brain area controlling many aspects of reproduction and behavior (Foran and Bass, 1999b). Although a potential linkage between these two hormones has been proposed in several vertebrate classes as a pathway connecting behavior and reproduction, evidence for a linkage, such as a hormone receptor, has not been conclusively demonstrated. The coupling of behavior and reproductive development is evident in many vertebrate groups where commensurate rises in aggression and courtship are seen with increases in gonadal development (Liley and Stacey, 1983). In the special case of hermaphroditic fishes that change sex from female to male, AVT associated sexual behavior can be de-coupled from the reproductive axis, which is under the control of GnRH, as demonstrated by persistence of sex-related behaviors even after removal of the gonads in bluehead wrasse (Semsar et al., 2001a). Therefore hermaphroditic fishes make excellent models to examine the relationship between AVT and GnRH between female and male phenotypes as well as changes in these two pathways during transition from a female to a male phenotype.

Sex changing fishes such as groupers are interesting model to study the AVT and GnRH pathways because of their ability to change sex, both behaviorally and gonadally

as adults. This condition allows for the examination of processes such as behavior and reproductive development during sexual change that would be difficult to study in gonochoristic (non-sex changing) fish. Behavioral differences and time period for sex change have recently been described for a small species of grouper, the rock hind, *Epinephelus adscensionis*, under captive conditions using a behavioral paradigm developed in our laboratory (Kline chapter 2). This work showed that rock hind behavior and gonadal sex can be altered via manipulations of the social environment, but data on differences in the AVT or GnRH systems between sexes or during sex change is currently lacking for this and other grouper species.

AVT and the homologous mammalian arginine vasopressin (AVP) have been associated with several physiological processes such as: seasonal and daily rhythms, osmoregulation, metabolism and the vasopressor response (see review by Balment et al., 2006). Moreover, the linkage of the AVT/AVP system with behavioral functions has received considerable attention regarding control of male-specific behaviors such as courtship and aggression (Goodson and Bass, 2000; Grober et al., 2002; Lema and Nevitt, 2004; Salek et al., 2002; Semsar et al., 2001a).

Among the sex changing species, the abundance of both AVT producing neurons in goby (Maruska et al., 2007b) and mRNA expression in bluehead wrasse (Godwin et al., 2000) are associated with sexual phenotype. These findings are also consistent with research on cichlid (*Astatotilapia burtoni*) (Greenwood et al., 2008) and pupfish (*Cyprinodon nevadensis amargosae*) as well as anurans *Acris crepitans* (Marler et al., 1999) and *Rana catesbeiana* (Boyd, 1997) that have sexually dimorphic preoptic AVT-producing cell populations. Sexually dimorphic AVT/AVP neuron populations have been associated with social behavior in many species from fish to birds and mammals. Exogenous treatments of AVT have yielded immediate changes in calling behavior and

territoriality in birds, frogs and fish and central administration directly to the brain has been successful in several studies (Boyd, 1991; Goodson and Bass, 2000; Maney et al., 1997) demonstrating that the behavioral effect likely originates from the brain rather than other areas of the body.

In general, three main types of AVT/AVP receptors exist in all vertebrates (see review by Balment, 2006). These receptors are named V1a, V1b and V2 and differ in their tissue expression and major known functions. Most behavioral effects of the AVT/AVP system have been attributed to the V1a receptor sub-type due to its high expression in the brain and particularly in the POAH and also due to the observed behavioral changes that occur when a V1a specific antagonist is used (Goodson and Bass, 2000; Propper and Dixon, 1997; Salek et al., 2002). Presently in fish, only two of the major forms, V1a and V2 have been identified and recently two cDNA sequences coding for two distinct forms of the V1a receptor have been described in pupfish (*Cyprinodon nevadensis amargosae*) (Lema, 2010) and are present in the fugu (*Takifugu rubripes*) GenBank database (see Table 1). The two V1a forms in pupfish show overlapping distributions in the forebrain, midbrain, cerebellum and hindbrain.

Evidence for a relationship between the AVT V1a type receptor and male typical behaviors has been reported in various vertebrate groups based on data obtained for the disruption of these behaviors by an AVP V1a-specific antagonist. These antagonist effects range from delayed aggression and marking behavior in hamster (Albers et al., 1986), to reduced courtship and aggression in bluehead wrasse (Semsar et al., 2001a), inhibition of male-specific mating behaviors in the newt (*Cynops pyrrhogaster*) (Goodson and Bass, 2001; Moore and Miller, 1983) and calling behavior in the Atlantic midshipman (Goodson and Bass, 2000) and toad (*Bufo cognatus*) (Propper and Dixon, 1997).

In fishes, gonadotropin releasing hormone (GnRH) plays several roles in reproduction and behavior. Multiple forms of GnRH occur and several of these have been implicated in behavior (Ogawa et al., 2006; Propper and Dixon, 1997). The form of GnRH from the preoptic area that is released from the nerve terminals in the teleostean pituitary and median eminence in mammals is considered the key hormone controlling reproductive function in all vertebrates (Gore, 2002). This form is typically identified as GnRH-I and the decapeptide hormone is identical in all Perciform fish studies to date (Lethimonier et al., 2004; Miranda et al., 1999; Mohamed et al., 2005; Senthilkumaran et al., 1999a). In groupers, three forms have been identified by their peptide isolation by HPLC (Nancy Sherwood, pers. comm.) and three cDNA sequences have recently been published for orange-spotted grouper (see Table 1). However, there are no published data regarding the distribution of GnRHs in the brain of any grouper species.

AVT and GnRH are known to co-vary in expression in both mRNA levels and protein levels according to season in the goldfish (Parhar et al., 2001) and across sexual phenotypes in the half-spotted goby (Maruska et al., 2007b). In addition, studies in fishes have noted the close proximity of some populations of AVT and GnRH, particularly in the parvocellular region (Maruska et al., 2007b; Saito et al., 2003). In chicken, it has been hypothesized that AVT acts to stimulate/induce release of GnRH due to the close proximity of neurons in the preoptic area (D'Hondt et al., 2000). In rat and baboon, *in-vivo* experiments provide evidence that GnRH release by AVP does in fact occur in the preoptic area but not in the pituitary (Koyama and Hagino, 1983; Salisbury et al., 1980). Blockage of V1a receptor by central administration of an antagonist in the preoptic area causes inhibition of LH secretion, implying a stimulatory influence of AVP on GnRH in mammals (Miller et al., 2006). Alternatively, evidence in trout and goldfish suggest that GnRH modulates AVT release due to the proximity of AVT and GnRH fibers and

increases in Ca^{2+} signaling of AVT-containing fibers when GnRH treatment is applied (Saito et al., 2003). However in these examples, a specific molecular mechanism involving receptor mediation has not been described.

In many fish species, the androgen 11-keto testosterone (11-KT) is the major male form, and in females E2 is the predominant sex steroid in reproductive fish (Devlin and Nagahama, 2002). In studies with sex changing groupers and wrasses, the hormonal profiles change dramatically with sexual phenotype, coincident with reorganization of the gonad and sex-specific coloration in males (Higa et al., 2003; Nakamura et al., 2007; Semsar and Godwin, 2004b). In both groupers and wrasses, the presence of circulating E2 has been demonstrated to inhibit the transition from female to male, even in the presence of high levels of 11-KT (Bhandari et al., 2005; Higa et al., 2003). Thus it is important to examine sex steroid profiles as a measure of gonadal stage when comparing AVT and GnRH systems in males versus females or during transition from a female to male phenotype.

The major objective of this research was to test the hypothesis that AVT can act on the GnRH system via a vasotocin V1a receptor to influence sexual behavior and reproductive development in a sex changing fish. The AVT, GnRH and two AVT receptor cDNAs were isolated and characterized, and calibrated assays were developed to measure levels of mRNA expressions using quantitative real time PCR (qRT-PCR). Immunohistochemistry (IHC) experiments were conducted to co-localize GnRH with the AVT V1a receptor in an attempt to identify a linkage between the AVT system and GnRH. Sexual differences that might be present in this sex changing fish and potential changes in the AVT, its receptors and GnRH mRNAs during the early stages of behavioral sex change were also examined. In addition, alterations in the AVT and GnRH

systems were evaluated in relation to sex steroid hormone levels between males and females and during the early stages of the sex change process.

METHODS

AVT receptor and GnRH immunohistochemistry

Two male rock hinds were anesthetized and their brains removed and processed for immunohistochemistry as detailed in Chapter 2. Brains were sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) in two series and stored at -80°C. Sections from the preoptic area were processed for GnRH and V1a2 receptor IHC for both DAB and fluorescence as follows: slides were removed from -80°C and stored in a vacuum dessicator for 30 min to equilibrate to room temperature and remove condensation. Sections were rehydrated in 1X PBS for 15 minutes then incubated in 1% hydrogen peroxide in PBS for 10 min. After washing 3 times in 1X PBS, antigen retrieval was performed by incubating in citrate buffer (10mM Citric Acid, pH 6.0) in a steamer for 15 minutes. After cooling and rinsing 3XPBS, sections were blocked for 1 hour in blocking solution (3% normal goat serum and 0.3% TritonX-100 in PBS). Blocking solution was removed and sections were incubated in affinity purified anti-rock hind V1a2 antibody (1:1k) or anti-sbGnRH antibody (1:10k; AS-691, Senthilkumaran et al., 1999) in PBS and slides were placed in a humidity chamber at 4 °C overnight. After incubation, sections were rinsed 3X over 45 min in PBS. Sections were then incubated for 2 hours with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories), rinsed again and incubated for 30 min with ABC reagent followed by peroxidase (Vector Laboratories) according to the manufacturer's instructions. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) substrate (impact DAB, Vector Labs). Sections were then

dehydrated with an alcohol series and cover-slipped with Cytoseal-60 (Fisher Scientific, Itasca, IL).

Double labeling for GnRH and V1a2 in rock hind preoptic area

Since both antibodies for GnRH and V1a2 were raised in rabbit, a double labeling protocol was utilized (Kroeber et al., 1998). Three hundred µl of affinity purified anti-RHV1a2 antibody was directly labeled with dylight-488 antibody labeling kit (#53024, Pierce Products, USA). Remaining unbound fluorophore was column extracted following the manufacturer's protocol.

Fluorescence IHC for GnRH followed the same protocol listed above for IHC with DAB until the incubation of the secondary antibody: Sections were incubated for 2 hours with a fluorescent rhodamine red-x conjugated goat anti-rabbit secondary antibody (1:500; Jackson immunoresearch) in the dark. Sections were then rinsed 3 X over 45 min in PBS. Sections were blocked with 5% normal rabbit serum (from the same rabbit that the anti RHV1a2 was raised in) in PBS-T to occupy any free binding sites for rabbit antibodies remaining on the secondary antibody. Sections were blocked for 1 hour in blocking solution (3% normal goat serum and 0.3% TritonX-100 in PBS). Blocking solution was removed and sections were incubated in directly labeled anti-RHV1a2-dylight 488 antibody (1:500) in PBS in a humidity chamber at 4 °C overnight in the dark. The following day, sections were rinsed in PBS 5X over 45 min, then dehydrated and cover-slipped in a darkroom. Fluorescence signal for 488 and rhodamine red-x was visualized on a Nikon eclipse C2 confocal microscope and imaging software. Specificity of both antibodies has been demonstrated previously for GnRH (Senthilkumaran et al., 1999a) and RHV1a2 (Kline, Chapter 3).

Isolation and characterization of cDNAs from rock hind brain

cDNA sequences were isolated and identified for development of quantitative real time PCR assays to quantify expression of AVT, two AVT V1a receptors and GnRH. Total RNA was extracted from whole brain of two rock hinds using Tri-reagent following the manufacturer's protocol (Molecular Research Center Inc. Cincinnati, OH) with a hand-held motorized pestle and tube homogenizer. Extracted RNA was quanitified using a nanodrop spectrophotometer (ND-1000) and RNA quality was estimated by visualizing intact 18S and 28S bands with 1% agarose gel electrophoresis with U/V transilluminator. RNA was stored at -80°C until use in cDNA synthesis reactions.

Five hundred µg RNA was processed to remove the 5' cap enzymatically and cDNA was reverse transcribed using an oligo (DT)18 adapter primer and the Generacer 5' cDNA synthesis kit following manufacture's protocol (Invitrogen, Carlsbad, CA). cDNA synthesis was performed at 50 °C for 1h then treated with 1U RNase-H at 37 °C for 30 min.

Transcripts for GnRH, AVT, and two distinct AVT V1a receptor sub-types were isolated using a protocol detailed in Mohamed et al. (2005). First and nested, forward and reverse primer pairs for each transcript (Table 2) were designed from consensus regions identified in alignments of closely related Perciform species identified in Genbank with the ClustalW program (Table 1). PCR was performed using Go-Taq green polymerase master mix (Promega, Sunnyvale, CA), 200nM of each primer and 1µl template cDNA with a PCR thermocycler (Eppendorf, Westbury, NY) and cycling conditions of 95°C for 2min, 35 cycles of 95°C for 40s, 58°C for 60s, 72°C for 60s, followed by 72°C for 7min and a holding temperature of 4°C. PCR products were separated by agarose gel electrophoresis and a band of the desired size was extracted using an extraction kit (Epoch Biolabs, Sugar Land, TX). PCR product extracts were ligated to PGEM T-Easy

vector plasmids and transformed into competent cells following manufacturer's instructions (Promega). Plasmid DNA was extracted and purified from bacterial culture (Epoch Biolabs) prior to sequencing in triplicate using T7 or SP6 universal primers.

NAME	SPECIES	ACCESSION #	FAMILY
GnRH			
Rock hind sbGnRH	*Epinephelus adscensionis*	HQ662335	Serranidae
Grouper GnRH-I	*Epinephelus brunneus*	FJ380048	Serranidae
Seabass GnRH-I	*Dicentrarchus labrax*	AF224279	Moronidae
Cobia sbGnRH	*Rachycentron canadum*	AAT80334	Rachycentridae
Tuna GnRH-I	*Thunnus thynnus*	ABX10867	Scombridae
Cichlid sbGnRH	*Astatotilapia burtoni*	AAC59691	Cichlidae
Seabream sbGnRH	*Sparus aurata*	AAA75469	Sparidae
Grouper GnRH-II	*Epinephelus coioides*	ACZ51152	Serranidae
Grouper GnRH-III	*Epinephelus coioides*	ACZ51151	Serranidae
AVT			
Rock hind AVT	*Epinephelus adscensionis*	ADO33898	Serranidae
Grouper	*Epinephelus coioides*	ADF36550	Serranidae
Flounder	*Platichthys flesus*	BAA98140	Pleuronectidae
Cichlid	*Astatotilapia burtoni*	AAM70492	Cichlidae
Pupfish	*Cyprinodon nevadensis*	ACZ01984	Cyprinodontidae
Wrasse	*Thalassoma bifasciatum*	AAN87838	Labridae
Sucker-VT-2	*Catostomus commersonii*	AAA49199	Catostomidae
Sucker-VT-1	*Catostomus commersonii*	AAA49198	Catostomidae
AVT Receptors			
Fugu V1b	*Takifugu rubripes*	AAK18744	Tetraodontidae
Pupfish V1a2	*Cyprinodon nevadensis*	ACX85729	Cyprinodontidae
Flounder V1a	*Platichthys flesus*	AAF00506	Pleuronectidae
White sucker VTR	*Catostomus commersonii*	CAA53958	Catostomidae
Rock hind V1a1	*Epinephelus adscensionis*	HQ662334	Serranidae
Rock hind V1a2	*Epinephelus adscensionis*	HQ141396	Serranidae
Pupfish V1a1	*Cyprinodon nevadensis*	ACX85728	Cyprinodontidae
Fugu V1a1	*Takifugu rubripes*	AAK17004	Tetraodontidae
Lungfish V1a	*Protopterus annectens*	BAG66063	Protopteridae
Newt V1a	*Cynops pyrrhogaster*	BAF38754	Salamandridae

Table 4.1. Protein sequences and GenBank accession numbers for rock hind and other species used for comparisons in this study.

Transcript	Primer name	Sequence (5'-3')
Pro-GnRH	GnRH-FW1	GGACTGAAGGAGTAGAAAGAGAC
	GnRH-FW2	GACTGAAGGAGTAGAAAGAGACG
	GnRH-RV2	TGTCCATTTCCCCTGTCGGT
	GnRH-RV1	TTGGCAAAAGGTGATTCCTC
Pro-AVT	AVTFW1	TACATCCAGAACTGYCCCCG
	AVT-FW2	TGYTACATCCAGAACTGYCC
	AVT-RV1	GGTGAGCAGGTAGTTCTC
	AVT-RV-2	CCAGGCAGTCAGAGTCCACC
V1a1 & V1a2	AVTr-FW1	AGCCTGGCBGAYCTGGTGGT
	AVTr-FW2	TTCCAGGTGCTSCCRCAGCT
	AVTr-RV1	ATCCASGGRTTRCAGCAGCT
	AVTr-RV2	CASGGRTTRCAGCAGCTGTT

Table 4.2. Oligonucleotide primer sequences used to isolate transcripts.

Gene-specific primers for use with 5' and 3' RACE adapter primers were designed from sequencing products that were identified as AVT, V1a receptor or GnRH sequences using NCBI blast search. PCR, cloning and sequencing was repeated as listed above and cDNA sequence fragments for each transcript with high overlap were used to construct full-length coding regions for GnRH, AVT, V1a1, and V1a2. These sequences were translated into protein sequences using the DNA to protein translation tool (www.biophp.org).

Amino acid translations for AVT, V1a1 and V1a2, and GnRH cDNAs were compared to protein sequences for other species selected from the NCBI database (Table 1) Alignments and % similarity scores for each transcript were made with the ClustalW program. For AVT and GnRH, translated protein components for signal peptide, hormone, processing sites, and associated peptides were inferred from other published sequences by alignment. For AVT V1a1 and V1a2 receptor sequences, transmembrane helices, intracellular, and extracellular loops were predicted using the MEMSAT3 program (Jones, 2007).

Phylogenetic analysis

Deduced amino acid sequences for the rock hind V1a1 and V1a2 receptor subtypes were aligned with other sequences in the vasotocin/ vasopressin, and oxytocin, mesotocin, and isotocin families, as well as, other similar receptor sequences from vertebrates and invertebrates. All sequences were first aligned with ClustalW program (Larkin et al., 2007). The phylogenetic tree and analysis was performed with the MEGA4 program (Tamura et al., 2007) using the Neighbor-joining method for phyology reconstruction, 1000 bootstrap replications for phylogeny tests, and the Poisson model for substitutions, Pairwise comparisons were used to address gaps in sequences.

Design of qRT-PCR assays and cRNA standard curves

Transcript	Primer name	Sequence (5'-3')	Amplicon size (bp)	PCR efficiency (avg%)
Pro-GnRH	RHSB-FW RHSB-RV	GGAGTAGAAAGAGACGCCTGC TTGGCAAAAGGTGATTCCTC	252	98.2%
Pro-AVT	AVT-FW AVT-RV	ATCCTGGGGGAGAGAAATCA GCACTGTCATTCGGCTGTAG	228	99.0%
V1a1	V1a1-FW V1a1-RV	CCATCTGGAGGAACCTGAAA GGAGAAGGTCTTGTCCCACA	224	97.5%
V1a2	V1a2-FW V1a2-RV	GAACAAGAACGGGCTGATTG CACACGATGTACGCCAAAAC	114	98.9%

Table 4.3. Oligonucleotide primer sequences used in quantitative real-time RT-PCR.

Primers for qRT-PCR assays were designed for each transcript by selecting a region that was distinctive for each transcript yielded only a single band of the expected size when separated by 2% agarose gel electrophoresis. Bands produced with PCR from candidate primer sets were extracted and directly sequenced to verify the presence of the desired transcript (Table 3). Synthesis of cRNA transcripts for use in qRT-PCR experiments was conducted using plasmids with a fragment of the coding region from gene specific primers. Plasmids for each transcript were linearized with Notl restriction

enzyme and cRNAs were synthesized using a T7 RNA polymerase kit following manufacturer's protocol (Promega). The synthesis reaction was purified by phenol-chloroform extraction and ethanol precipitation, then reconstituted in nuclease free water and treated with 1U DNAse. The cRNAs were quantified with a spectrophotometer at 260nm (Nanodrop ND-1000). Each standard RNA copy number was estimated with the formula: $N = C \times 6.022 \times 10^{23}/MW$, where N = molecules per µl, C = cRNA (g/µl), and MW = molecular weight.

One-step SYBR green qRT-PCR assay conditions were optimized for each transcript primer set by modifying the temperature of the RT step, temperature and time periods for the annealing and extension points in each cycle to achieve the highest efficiency (Table 3), fluorescence values and a single peak at the predicted temperature for each transcript in the final melting curve. Cycling conditions were set for 37 cycles for each transcript. A no-template control (NTC), and a no reverse transcriptase (no-RT) well were included in each assay, in duplicate. A single clear peak was observed in each dissociation curve for the standard curves and samples analyzed in this study. A standard curve with five steps and ten-fold dilution at each step was used to calibrate each assay to copies per ng.

Quantification of mRNA transcripts for AVT, AVT V1A1 and V1A2 receptors, and GnRH was performed using one-step qRT-PCR (Stratagene) with SYBR-green in duplicate 25µl reactions using 75-150 ng total RNA. Total RNA was measured on a nanodrop for dilution; then measured by fluorescence, in duplicate using the RNA quant-it kit (Molecular probes). Values from qRT-PCR experiments were scaled to a cRNA standard curve and normalized to total RNA input. At the end of each assay, a disassociation curve was used to confirm that only one peak was observed at the predicted melting temperature for a particular transcript. In the rare case that samples had

more than one peak in a melting curve, samples were re-run or discarded. PCR products were separated on 2% agarose gel electrophoresis to verify the presence of a single band of the expected size.

Brain dissection and region distribution of mRNAs

Rock hind brain was dissected into seven regions: olfactory bulbs (OB), telencephalon (TEL), preoptic area and hypothalamus (POA+H), pituitary (PIT), cerebellum (CE), optic tectum (OT), inferior lobes (IL), medulla oblongata (MO) with the aid of a rock hind brain atlas (Kline chapter 3). POA+H dissection captured the majority of expression of AVT, V1a1 and V1a2 receptors, and GnRH in this region, which corresponds to slides C-L in Figure 2 (Kline chapter 3). RNA was extracted for later use in qRT-PCR experiments as detailed previously.

Sampling for male versus female comparison and behavioral experiments

Male and female rock hind were collected from oil production platform sites in the Gulf of Mexico near Port Aransas, TX in May-June 2008. After a one-week recovery period, sexes were determined as detailed in Kline chapter 2. Rock hind were placed in tanks ranging in size from 1300 to 4000 l with a single structure made of 10cm PVC as detailed in Kline chapter 2. After a four week stabilization period, seven male and twelve female rock hind were sampled from these established social hierarchies. In subsequent time series experiments, dominant male rock hind were removed after the stabilization period and the largest remaining female and a smaller female were sampled at 3d and 10d for five replicates at each time period. In three groups at the 10d sampling period medium sized females were collected as well for comparison.

For sampling of blood plasma, brain and gonad tissues, fish were placed in a bath of clove oil (0.1ml/l) then body weight and total length were recorded. Blood was drawn

from the caudal vein by a heparinized syringe and plasma samples separated by centrifugation at 2500G for 20 min at 4 °C were and stored at -20°C until use in steroid hormone assays. Rock hind were killed by decapitation, brains removed and placed in RNA Later solution (Ambion, Austin, TX) on ice, stored at 4°C overnight then stored at -20°C until processing. Gonads were removed, weighed to the nearest g and preserved in 10% buffered formalin. Brains were dissected into seven regions and POA+H region processed for qRT-PCR experiments as listed above. Plasma 11-KT and E2 levels were extracted and measured by ELISA (as detailed below).

All brains and tissues were preserved in RNA later solution until extraction. Total RNA was extracted using Tri-reagent (MRC). After extraction, RNA was treated with 1U/ μgDNAse (Promega). RNA quality was estimated by the ratio of absorbance at 260nm to 280nm (260/280) as well as visualizing intact 18S and 28S bands on an 1% agarose-ethidium bromide gel with U/V transilluminator. RNA was stored at -80°C until use in cDNA synthesis reactions.

Plasma steroid measurements

One hundred μl of rock hind blood plasma was extracted for each sample. Plasma was diluted in 1ml Tris-saline buffer (20 mM Tris, 0.9% NaCl, pH 7.4), vortexed, then centrifuged at 15,000G for 15 minutes to remove clots and cellular debris. Diluted plasma was double extracted in 5ml diethyl ether and evaporated in a vacuum centrifuge. Extracted samples were diluted as calculated from original plasma volume in EIA buffer (100 mM phosphate, 0.1% BSA, 400 mM sodium chloride, 1 mM EDTA and 0.01% sodium azide) 1:30 for 11-KT and 1:8 for E2. Steroid ELISAs were performed according to manufacturers' protocol (Cayman Chemical, 11-KT: Alpha Diagnostics, E2). Absorbance was measured on a spectrophotometer after overnight incubation for 11-KT

and 2 h incubation for E2. Steroid measurements were multiplied by the dilution factor for % recovery for each assay. Percent recovery was 97% for 11-KT and 102% for E2 as measured for steroid spiked samples.

Statistical analyses

Comparison of values for various assays in male versus female rock hind was performed using t-test for unpaired samples. ANOVA on ranks (Kruskall-Wallis) followed by a Dunn's post-hoc test were used for multiple comparisons in the time series experiments as compared to initial female values. Linear regression was used to compare trends seen in transcript values and steroid hormones. Mean and standard errors are reported for all values.

RESULTS

Immunohistochemistry and co-localization of AVT V1a2 and GnRH

Immunohistochemistry with DAB staining for GnRH and V1a2 receptor in serial sections of the pre-optic area in rock hind showed distinctive neurons and fibers labeled for GnRH (Fig. 1A) and cells identified in the parvocellular region labeled for the V1a2 receptor (Fig. 1B). Co-localization of GnRH and the AVT V1a2 receptor in the same sections showed neurons and fibers labeled for GnRH (red) with primarily membranes of the same cell bodies and those of other unidentified cells labeled for AVT V1a2 receptor (green) (Fig. 2A-B). This co-localization was observed in GnRH cell populations within the parvocellular region of the preoptic nucleus that had fibers that led to the pituitary, thus confirming that a pathway could exist for AVT to act on GnRH neurons.

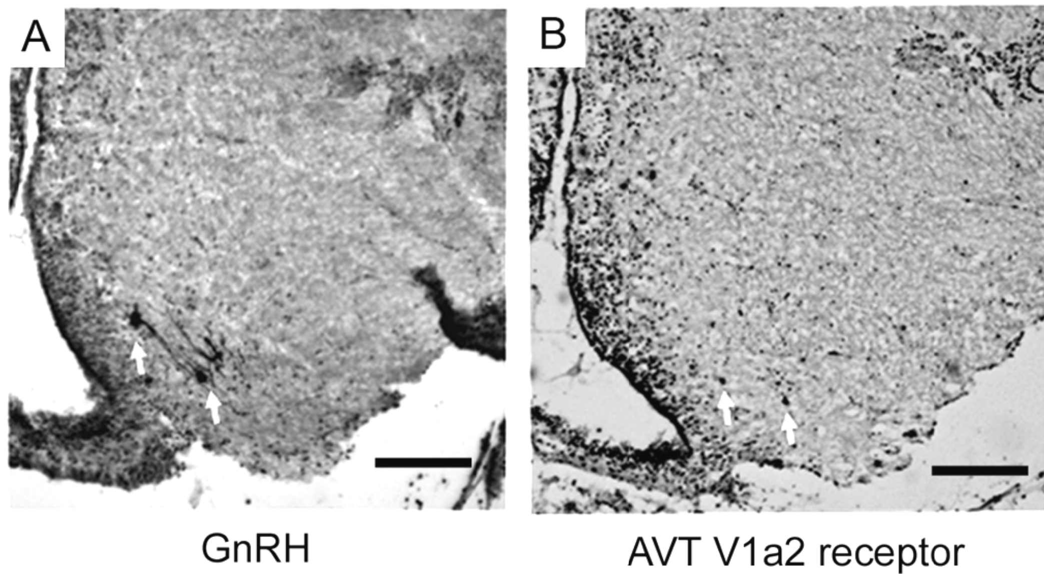

Figure 4.1. Adjacent 20μm cross-sections of rock hind preoptic area identifying GnRH producing cells (A) and AVT receptor (B) in the preoptic parvocellular region (white arrows). Immunohistochemistry used:10,000 anti-sbGnRH (A) and 1:1000 anti-rock hind V1a2 (B) antibodies followed by 1:300 anti-rabbit HRP secondary antibody and labeled with DAB staining.

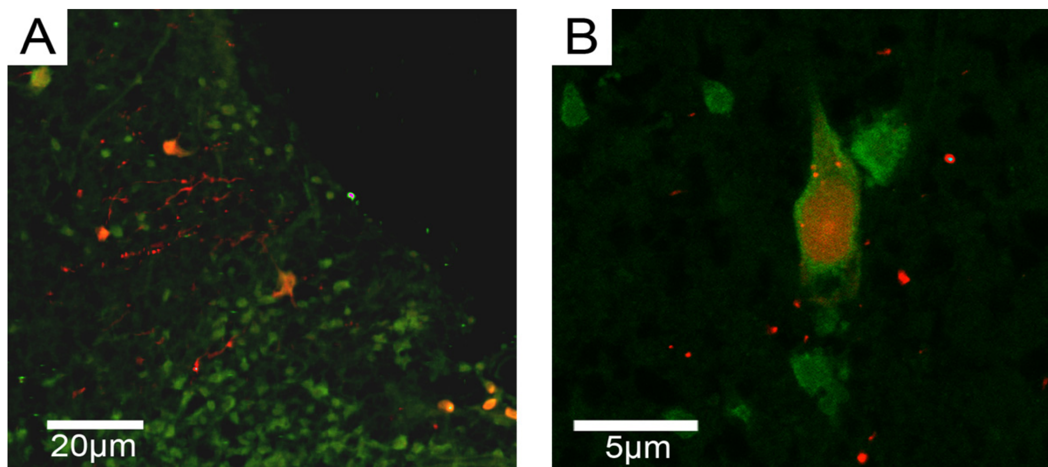

Fig. 4.2. Immunohistochemical co-localization of AVT V1A2 receptor on GnRH producing neurons in the parvocelluar preoptic area of rock hind brain. Low magnification (A) shows GnRH fibers and cell bodies with only cell bodies labelled with for the V1a2 receptor and high magnification (B). Primary antibodies for GnRH (1:10,000) and secondary labeled antibody (red) (1:1000); were used with directly labeled antibodies for the AVT V1a2 receptor (green)(1:1000).

Characterization of rock hind AVT, V1a1 and V1a2 receptors and GnRH

Translation of the pre-pro AVT mRNA sequence yielded a 153 polypeptide sequence identical in size to five other published sequences in Perciform fish (Fig. 3). The sequence region for AVT hormone and the processing and amidation sites were identical for rock hind AVT and the seven other sequences from fishes compared. The complete AVT sequence for orange-spotted grouper had 90% similarity to that for rock hind and those of other fish species compared were 80-86% similar to rock hind. In white sucker (Cypriniformes), two AVT forms have been identified and both sequences were

```
             Signal Peptide            PAS
                 +-----------------+ Hormone +-+       Neurophysin
                                   +-------+   +--------------------------
Rock hind       -MPHSLFPLCVLGLLTLSSACYIQNCPRGGKRALPETGIRQCMSCGPGDRGRCFGPSICC 59
OS-Grouper      -MPHSMFPLCVLGLLTLSSACYIQNCPRGGKRALPETGIRQCMSCGPGDRGRCFGPSICC 59
Flounder        -MPHSMFPLCVLGFLALSSACYIQNCPRGGKRALPDTGIRQCMPCGPGDRGRCFGPGICC 59
Cichlid         -MPHSLFPLCVLGLLAFSSACYIQNCPRGGKRALTETGIRQCKSCGPGDRGRCFGPSICC 59
Pupfish         -MHHCLLPLCVLGLLALSSACYIQNCPRGGKRALPEGGIRQCMSCGPGDRGRCFGPSICC 59
Wrasse          -MPHSVIPLCVLGLLALSSACYIQNCPRGGKRALPETGIRQCMPCGPKDKGRCFGPNICC 59
Sucker-2        MSVCAVLLLCVAGLLCLSSACYIQNCPRGGKRALLEPVSRQCLACGPGDKGRCLGPSICC 60
Sucker-1        -MSDSFLPTCILCLLALSSACYIQNCPRGGKRSQPDT-SRECVSCGPGNAGRCYGPSICC 58

              -------------------------------------------------------------
Rock hind       GEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGLCCNSESCVVDPDCLG 119
OS-Grouper      GEGLGCLLGSPETAHCVEENYLLTPCQAGGRPCGSEGGRCAASGLCCNSESCMVDPDCLG 119
Flounder        GEGLGCLLGSPETAHCVEENYLLTPCMAGGRPCGSEGGRCAASGLCCNPESCAVDSDCLA 119
Cichlid         GDGFGCLLGSLETAHCVEENYLLTPCQAGGRSCGSEGGHCAISGFCCNSEGCMVDSDCLG 119
Pupfish         GEGLGCLLGSPASAHCEEENYLLTPCQAGGRPCGPEGGHCASSGLCCNTEGCMVDSDCLG 119
Wrasse          GEGLGCLLGSPETAHCLEENYLLTPCQTGGRPCGSEGGHCAAPGVCCNSESCAVDSDCLG 119
Sucker-2        GEEIGCLVGSPWMARCQEEEYLPSPCQTAGKLCGSDAGPCAAPGVCCGTEGCKLDPNCSE 120
Sucker-1        GAALGCLVGSPETMSCMEENHLPSPCETGGRPCGDE-GRCAAPGVCCDSVSCVMDSECLE 117

              ----------------------------------+
Rock hind       DTEASDPA-HGSAGSSPAELLMRLLHVATRGQTEY 153        Similarity
OS-Grouper      DTEASDPA-HGSAGSSPAELLMRLLHVATRGQTEY 153           98%
Flounder        EIEASDPG-HGAGGSSPAALLLRLLHVTARGQTEY 153           86%
Cichlid         ETEATDPV-HGSARSSPTELLMRLLHVAARGQNEY 153           86%
Pupfish         ETDATDPS-QSSSRNSPTDLLLRLLHVASRGQSDY 153           86%
Wrasse          EIEVSDQA-DSSAGSSPAELLLRLLHVATRGQTEY 153           80%
Sucker-2        DSESEEPADQNTLGASPGELLLRLLHPNNRKHNQY 155           59%
Sucker-1        DVRSDQSEDPSRLKTVSGEILLRLLNLASRGRRDF 152           59%
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Figure 4.3. Amino acid alignment and percent similarity of rock hind prepro-AVT and AVT amino acid sequences from other fish species and two sub-types reported in sucker. Amino acids with at least four matches in other species are highlighted in grey. Homologous regions for signal peptide, hormone, processing and amidation site (PAS) and leucine rich core (LRC) are identified. Genbank accession numbers are listed in Table 1.

only 59% similar to the rock hind AVT. In the neurophysin sequence all cysteine positions, thought to be important in the secondary structure, were conserved. Also, the leucine rich core region at the 3' end of the neurophysin was highly conserved between rock hind and the other species compared.

Based on the cDNA sequences isolated in rock hind for the V1a1 and V1a2 receptors, we determined that the translation yielded sequences of 413 and 384 amino acids, respectively. Alignment of the two receptor sequences with additional sequences from other species is presented in Figure 4. Percent similarity for rock hind V1a1 and V1a2 compared to other published sequences is presented in Table 4. Rock hind V1a1 receptor sequence was most similar to rock hind V1a2 and flounder V1 receptor sequences at 77 and 78%, respectively. However in fugu and pupfish, the only species in which two V1a receptor subtypes have been identified, the rock hind V1a1 and V1a2 receptor subtypes were most similar to the those types identified in fugu and pupfish. Published sequences for cichlid (*Astatotilapia burtoni*), flounder (*Platichthys flesus*), sucker (*Catostomus commersonii*), lungfish (*Protopterus annectens*) and newt (*Cynops pyrrhogaster*) V1 receptors all showed the highest similarity to the rock hind V1a2 receptor sequence.

Phylogenetic analysis

A phylogenetic comparison of the deduced amino acid sequences for AVT V1a1 and V1a2 for rock hind in this study compared with other sequences in the vasotocin/ vasopressin, and oxytocin, mesotocin, and isotocin families, as well as, other similar receptor sequences from vertebrates and invertebrates is shown in Figure 5. This phylogenetic comparison showed that the receptor subtypes identified in rock hind are most closely related to the V1a type receptor forms in other vertebrates. Furthermore, the

V1a subtypes cluster with other subtypes identified in pupfish and fugu. The other receptor families clustered into four distinct groups and no sequence identified in fish was grouped with the V1b subtype.

Modeling of the transmembrane helices, and intracellular and extracellular loops using the MEMSAT3 receptor protein modeling program predicted seven transmembrane domains for both the V1a1 and V1a2 rock hind receptor sequences. Alignment with other receptor sequences revealed high homology between the two receptor forms and the transmembrane domains (Fig. 4). Regions near the C-terminus and the N-terminus had the least homology as well as the third intracellular loop sequence, which had a 3 amino acid difference in V1a2 receptor compared to the V1a1 receptor sequences. The third intracellular loop (ICL III) is the location of the antigenic site for the RHV1a2 antibody as well as that used in a study on European flounder (Warne, 2001) and the sequence in this location differs substantially from the V1a1 form (See Figure 4 indicated with dollar signs).

```
                                                                                    TM I
Rock hind V1a2         --------------------------METPG---SNTVHPNGSDPFARNEEVAQIEIMVLSITFVVAVIG  41
Fugu V1a2              -MHTPDYALLLNGVNGSLVSRPIELNMGTSGNS-TELLNSNGSDPFARNEEVAQIEIMVLSITFVVAVIG  68
Pupfish V1a2           -------------------------MMGTPS---NSTVHPNGSDPFARNEDVAQIEIMVLSITLVVAVIG  42
European flounder      --------------------------MEKPG---NITLHPNGSDPFGRNEEVAQIEIMVLSITFVVAVIG  41
White sucker AVTr      --------------------------MGRIA---NQTTASNDTDPFGRNEEVAKMEITVLSVTFFVAVIG  41
Rock hind V1a1         MLCARDGSCNMTDTNCSDGFNLTHQPQPAATAA-NIIGRRNDTDPFGRNEEVAKLEITVLSLAFVAAVVG  69
Pupfish V1a1           MLCSSEISCNITEVFN----LTQKQEIGAAGSARNLSGKTNDTDPFGRNEEVAKIEIТVLSLAFVAAVVG  66
Fugu V1a1              ------------------------MLLSNSTEEPGANLSRNHTDPFGRNEEVAKIEIGVLSLTFVAAVLG  46

                                 ICL I        TM II                ECL I         TM III
Rock hind V1a2         NVSVLLAMYNTKKKMSRMHLFIKHLSLADLVVAFFQVLPQLCWKITYRFYGSDFLCRIVKHLQVMGMFAS  101
Fugu V1a2              NVSVLLAMHNTKKKISRMHLFIKHLSLADLVVAFFQVLPQLCWEITFRFYGSDFLCRIVKHLQVMGMFAS  128
Pupfish V1a2           NVSVLLAMYNTKKKMSRMHLFIKHLSLADLVVAFFQVLPQLCWKITDRFYGTDALCRIVKHLQVMGMFAS  102
European flounder      NVSVLLAMYNTKKKMSRMHLFIKHLSLADLVVAFFQVLPQLCWEITYRFFGPDFLCRIVKHLQVTGMFAS  101
White sucker AVTr      NLSVLLAMHNTKKKSSRMHLFIKHLSLADMVVAFFQVLPQLCWEITFRFYGPDFLCRIVKHLQVLGMFAS  101
Rock hind V1a1         NVSVLLAMYKTRRKPSRMHLFMKHLSLADLVVAVFQVLPQLCWEITFRFYGPDFLCRIVKHLQVLGMFAS  129
Pupfish V1a1           NLSVLLAMYRSRRKLSRMHLFMKHLSLADLVVAFFQVLPQLCWEVTFRFYGPDFLCRIVKHLQVLGMFAS  126
Fugu V1a1              NVSVLLATHR---KPSRVHLFMKHLSLADLVVAFFQVLPQLCWEVTFRFYGPDFLCRIVKHLQVLGMFAS  103

                                    ICL II                TM IV               ECL II
Rock hind V1a2         TYMMVMMTVDRYIAICHPLKTLQQPTQRSYIMIISTWMCSLVFSTPQYFIFSLSEIKNGSKIEDCWAHFI  161
Fugu V1a2              TYMMVMMTLDRYIAICHPLKTLQQSSQRSYIMIISTWMCSLALSTPQYFIFSLSEIEKGSEVYDCWAHFI  188
Pupfish V1a2           TYMMVMMTLDRYIAICHPLKTLQQSTKRSYVMIISTWICSLVLSSPQYFIFSMSEIKNGSEVYDCWAHFI  162
European flounder      TYMMVMMTLDRYIAICHPLKTLQQPTQRSYIMIVSTWMCSLVFSTPQYFIFSLSEVKNGSTVKDCWAHFI  161
White sucker AVTr      TYMMVMMTLDRYIAICHPLKTLQQPTQRAYIMIGSTWLCSLLLSTPQYFIFSLSEIQNGSYVYDCWGHFI  161
Rock hind V1a1         TYMMVMMTLDRYIAICHPLQTLQQPTQRAHIMIGSTWACSLVLSTPQYFIFSLSEVHPGSGVHDCWAHFM  189
Pupfish V1a1           TYMMVMMTLDRYIAICHPLQTLQQPTRRAYIMISSTWACSLVLSTPQYCIFSLSEVRPGSAVYDCWGHFV  186
Fugu V1a1              AYMMVMMTLDRYIAICHPLQTLQRPAQRAYVMIGGTWAGSLALSAPQYFIFSLSEVSPGSAVYDCWGHFV  163

                                     TM V                  $$$$$$    $$$$$$$$$  ICL III
Rock hind V1A2         EPWGAKAYITWITVGIFLVPVVILVMCYGFICHSIWNNIKYKKR---KSTAGAANKNGLIGKHSVSSITT  221
Fugu V1a2              EPWGAKAYITWITVGIFLVPVTMLMMCYGFICHSIWKNIKFKKR---KGTTGAATKNGLIGKNSVSSITT  248
Pupfish V1a2           EPWGSKAYITWMTVGIFLVPVVILILCYGFICHSIWKNIKYKKK---KTVAGAAGKNGLIGKCSVSSITT  222
European flounder      EPWGARAYITWITGGIFLVPVVILVMCYGFICHTIWKNIKYKKR---KTIPGAASKNGLIGKNSVSSVTT  221
White sucker AVTr      EPWGIRAYITWITVGIFLIPVIILMICYGFICHSIWKNIKCKTM---RGTR--NTKDGMIGKVSVSSVTI  221
Rock hind V1a1         EPWGLRAYITWMTAGIFLVPVAVLVFCYGLICRTIWRNLKYKTRT--RRTVAEANRSGMLGRSSVSSVST  249
Pupfish V1a1           EPWGPRAYITWITAGIFLVPVAVLVFCYGFICRAIWRNLKCKTRRKSADAVVEATKSGILGRSSVSSVST  246
Fugu V1a1              EPWGLRAYITWMTAGIFVVPVAALVFCYGFICRTIWKNLKCKTQRKSVEAVAEATGAGILGPCSVSSVST  223

                                   TM VI                       ECL III          TM VII
Rock hind V1a2         ISRAKLRTVKMTFVIVLAYIVCWAPFFTVQMWSVWDKNFQWVDSENTAVTLSALLASLNSCCNPWIYMIF  278
Fugu V1a2              ISRAKLRTVKMTFVIVLAYIVCWAPFFTVQMWSVWDENFQWADSENTAVTLSALLASLNSCCNPWIYMIF  305
Pupfish V1a2           ISRAKLRTVKMTFVIVLAYIICWAPFFTVQMWSVWDKNFKWDDSENTAVSLSALLASLNSCCNPWIYMIF  279
European flounder      ISRAKLRTVKMTFVIVLAYIICWAPFFTVQMWSVWDENFQYADSENTAVTISALLASLNSCCNPWIYMIF  278
White sucker AVTr      ISRAKLRTVKMTLVIVLAYIVCWAPFFIVQMWSVWDENFSWDDSENAAVTLSALLASLNSCCNPWIYMLF  276
Rock hind V1a1         ISRAKLRTVKMTFVIVLAYVVCWAPFFTVQMWSVWDETFSWHDSENTTVTLTALLASLNSCCNPWIYMIF  307
Pupfish V1a1           ISRAKLRTVKMTFVIVVAYVVCWAPFFTVQMWSVWDKTFSWDDSENTAVSLSALLASLNSCCNPWIYMTF  306
Fugu V1a1              LSRAKLRTVKMTFVIVLAYVLCWAPFFTVQMWSVWDHTFSWDDSESTAVTLSALLASLNSCCNPWIYMLF  283

Rock hind V1a2         SGHLLQDFVHCFSCCYKMNTDFKKEDSDSSLRRTTLLTKMTN-RSPTGSSSNWRELDNSPKSSIQTE---  384
Fugu V1a2               SGHLLQDFVHCLPCCVKMNTDFKKEDSDSSLRRTTLLSKMTN-RSPTASSGNWRELDNSPKSSNEVE---  411
Pupfish V1a2           SGHLLQDFMHCFSCCRKLNADYKKEDSDSSIRRTTLLTRITN-RSPTGSSSNWRELDNSPKTSAQTE---  385
European flounder      SGHLLQDFMNCFAWCRRANADFKKEDSDSSIRRTTLLTKMTN-RSPTGSTGNWRDLDNSPKTSIQME---  384
White sucker AVTr      SGHLLYDFLRCFPCCKKPRNMLQKEDSDSSIRRNTLLTKLAAGRMTNDGFGSWRDPCNSRKSSQSIG....434
Rock hind V1a1         SGHLLQDFMHCFSCCYRLNTDFKKEDSDSSIRRTTLLSKMTN-RSPTGSTSNWRELDNSPKTSAQTE---  413
Pupfish V1a1           SGHLLQDFMHCFSCCYRLRDKLKKQDSDSSIRRTTLLSRLQGPRLSKPFRELNYTIKNCPQATPAS----  412
Fugu V1a1              GGRLLSDCAGSLPCCSRLGRRFNCSFHILTTLFFLVGKEITGLLSLSCSQRTFKAKQDRGGGQNQTR....447
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Figure 4.4. Alignment of the V1a1 and V1a2 amino acid sequences identified in rock hind compared with other teleost species. Positions with four or more identical amino acids are highlighted in grey. Theoretical transmembrane domains (TM I-VII), and intracellular (IC I-III) and extracellular (ECL I-III) loops predicted fromMEMSAT3 are also identified. The antigenic site of the rock hind and flounder V1a2 receptor antibodies is also identified and differs from V1a1 substantially. Genbank accession numbers are listed in Table 1.

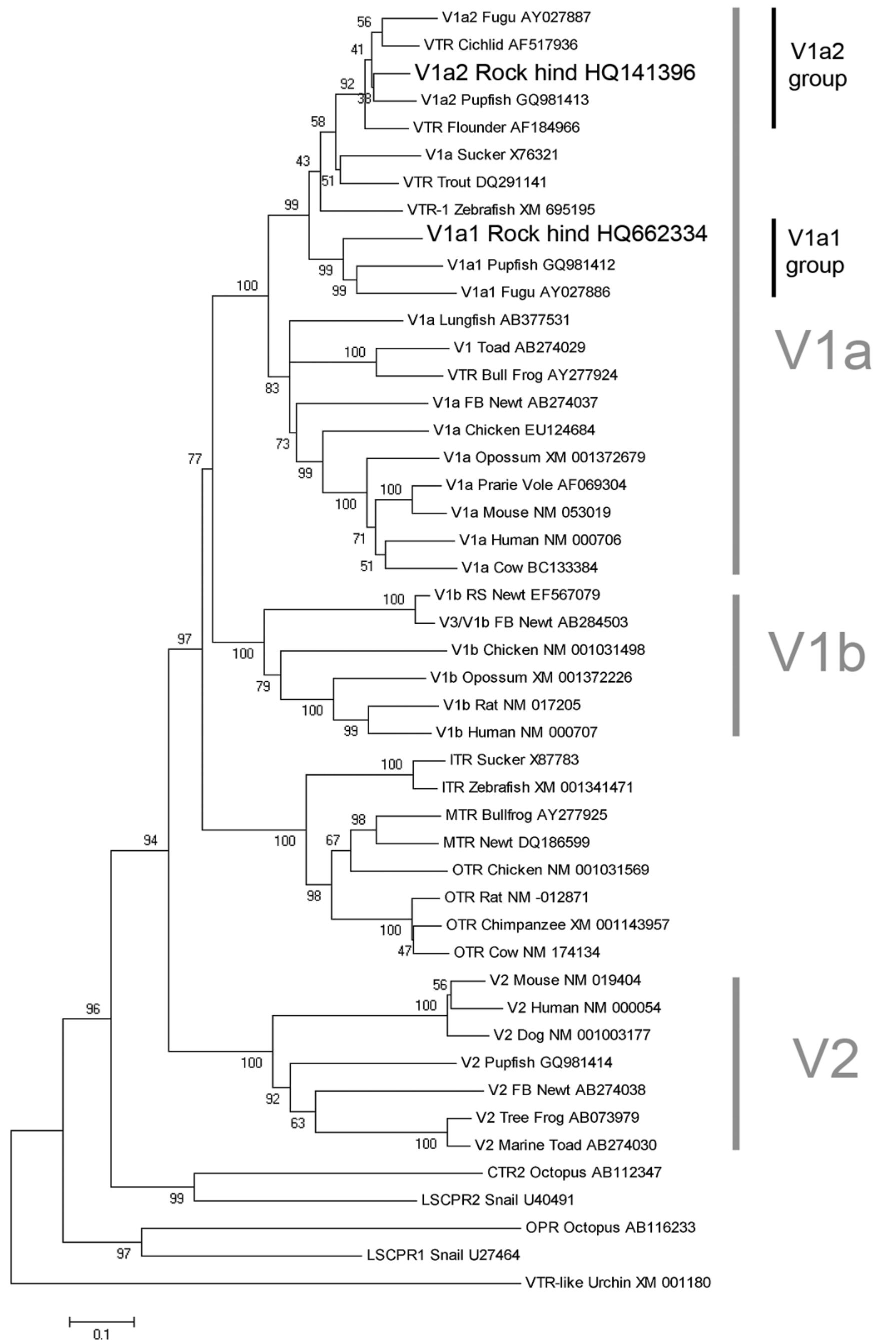

Figure 4.5. Phylogenetic tree comparing rock V1a1 and V1a2 with the VP/VT IT/MT family of receptors and invertebrate out-groups. Grey lines show the relationships of the three AVT/AVP receptor forms (V1a, V1b and V2). Two distinct V1a groups (V1a1 & V1a2) shown by black lines have been recently identified in fish and the V1a subtypes identified in this study for rock hind are highlighted in black. See Lema (2010) for a complete phylogenetic analysis.

Translation of the cDNA sequence for rock hind pre-proGnRH yielded a 94 amino acid sequence that was similar in size to other published sbGnRH sequences in fish (Fig. 5). Sequence similarity for the GnRH hormone and the processing and amidation site regions was 100% for all sbGnRH-type sequences compared. The highest similarity of the complete rock hind sequence was with the long tooth (LT) grouper (Epinephelus brunneus) at 97% and Bluefin tuna (Thunnus thynnus) at 76%. Two other

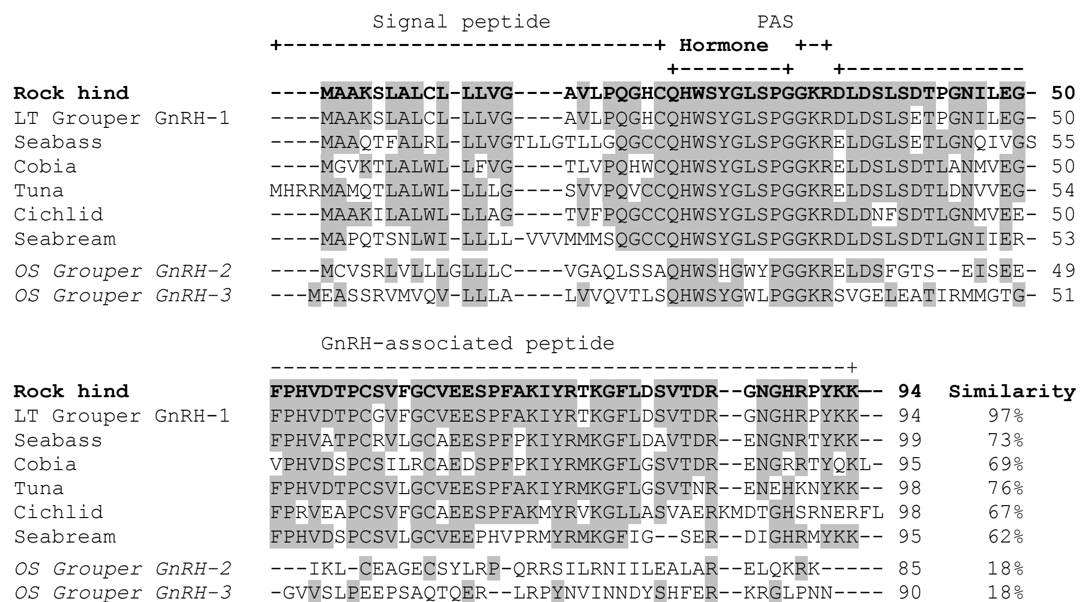

Figure 4.6. Amino acid alignment and percent similarity of rock hind prepro-GnRH-I and GnRH amino acid sequences from other fish species. GnRH-I sequences and two other GnRH types from (OS) orange-spotted grouper. Amino acids with at least four matches in other species are highlighted in grey. Homologous regions for signal peptide, hormone, processing and amidation site (PAS), and GnRH-associated peptide are identified. Genbank accession numbers are listed in Table 1.

forms of GnRH identified in orange-spotted (OS) grouper (Epinephelus coioides) for salmon-type GnRH (GnRH-III) and chicken II-type GnRH (GnRH-II) were only 18% similar to the sbGnRH sequence identified for rock hind. Although other forms have not been isolated in rock hind, the region used to design primers for use in qRT-PCR assays

differed substantially from the GnRH-II and GnRH III cDNA sequences published for orange-spotted grouper (57 and 49%, respectively).

The rock hind brain tissue distribution for AVT, GnRH, V1a1 and V1a2 mRNAs as measured by qRT-PCR and scaled to copies per 100 ng total RNA relative to cRNA standard curves demonstrated that the majority of transcripts for AVT and GnRH were captured in the preoptic area and hypothalamic region (POA+H). The POA+H also had the highest levels of expression for the V1a1 and V1a2 receptor mRNAs, compared to other regions dissected. Therefore this region was used for comparisons in subsequent experiments in this study. In addition to POA+H, GnRH mRNA expression was observed in the IL, PIT, CCe, and MO (Fig. 7). Additional expression for AVT was observed in the OB+TEL and MO. TheV1a1 and V1a2 mRNAs were detected also in the OB+TEL, IL, OT, CCe, and MO, whereas only V1a2 mRNA expression was observed in the pituitary.

Male versus female comparison

Comparisons of plasma steroids and POA+H mRNA transcripts between male and female rock hind are presented in Figure 7. Males had significantly higher levels of 11-KT than females with males having 12.1±1.4 and females having 2.3±0.3 ng/ml, respectively (t-test; $p<0.001$). Males had significantly lower E2 levels compared to females with males having 3.0±0.2 and females having 8.1±2.8 ng/ml) (t-test; $p=0.04$). However, female E2 levels were highly variable and corresponded to GSI levels (data not shown).

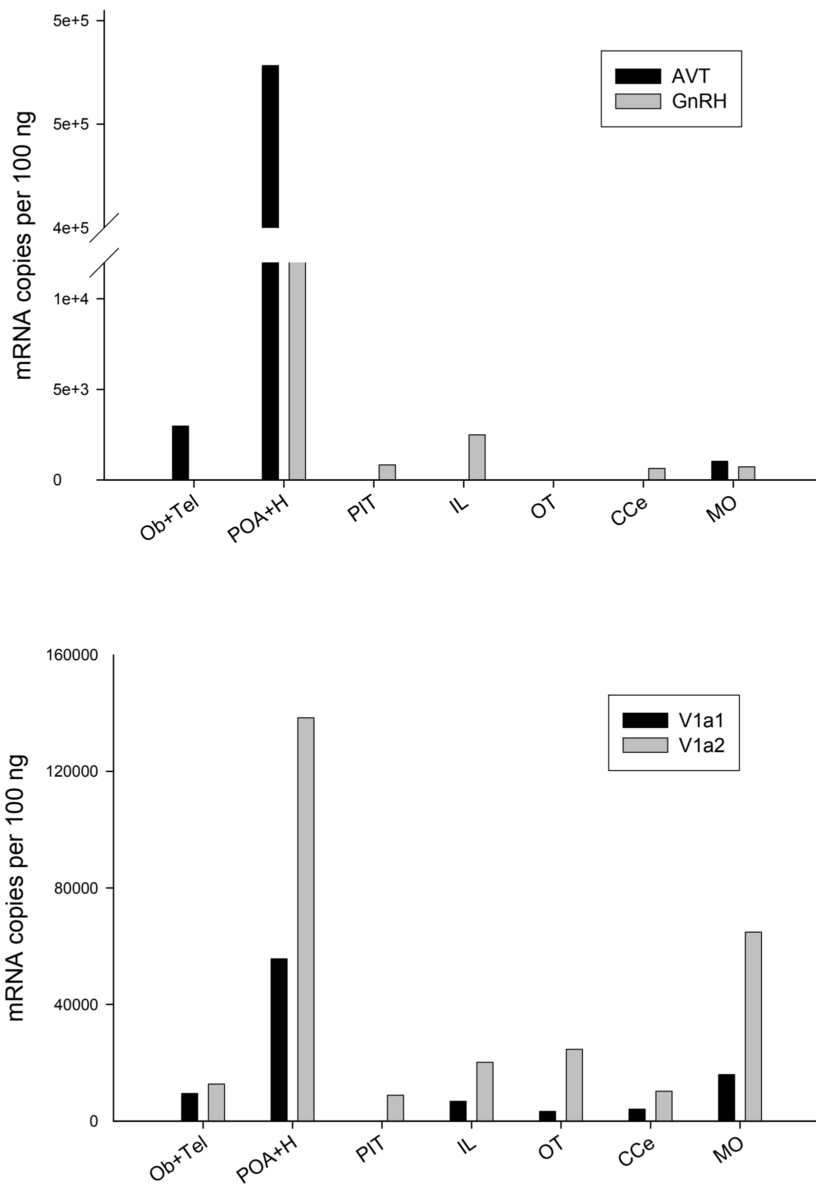

Figure 4.7. Brain distribution AVT, GnRH, and V1a1 and V1a2 mRNA transcripts. Levels were measured by qRT-PCR using cRNA standard curves for olfactory bulbs and telencephalon (OB+TEL), preoptic area+hypothalamus (POA+HYP), pituitary (PIT), inferior lobes (IL), optic tectum (OT), cerebellum (CCe), and medulla oblongata (MO) in copies per 100ng total RNA.

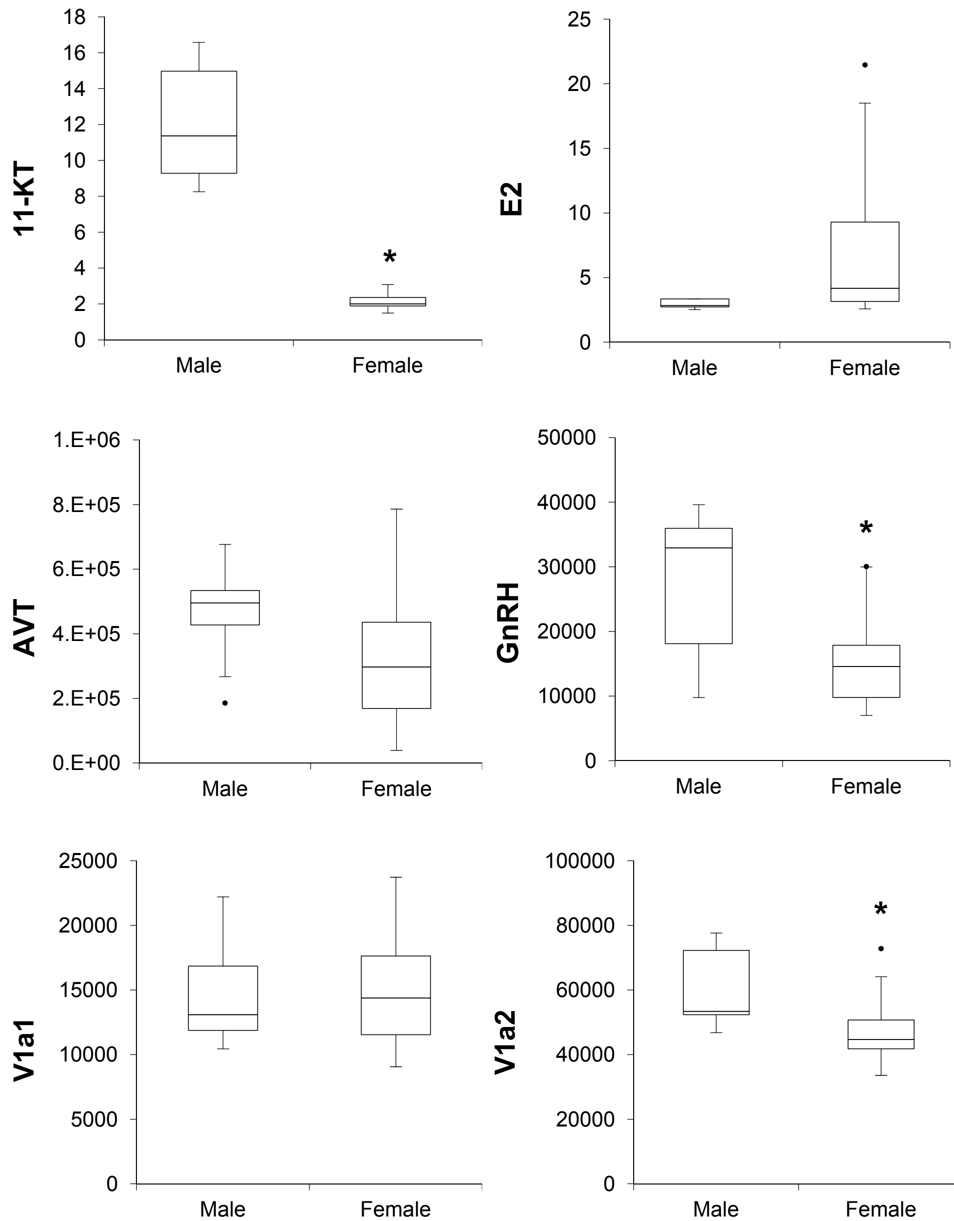

Figure 4.8. Comparison of steroid hormone and mRNA transcript levels between male and female rock hind. 11-keto testosterone (11-KT) and estradiol (E2) (in ng/ ml plasma) and mRNA transcript levels for AVT, GnRH and V1a1 and V1a2 receptors (in copies/ 100ng total RNA) from the preoptic area+hypothalamus of male and female rock hind. Social groups were stable for four weeks before sampling. N=7 and 13 for males and females, respectively. An "*" denotes significance at $p<0.05$ as measured with t-test.

AVT mRNA transcript levels were not significantly different between male and female rock hind (4687±580 and 3302±691 per ng total RNA, respectively). AVT mRNA levels in females were positively correlated with their GSI r^2=0.74 but not in males r^2=0.2. GnRH mRNA transcript levels were significantly higher in males than females with GnRH values of 272±45 and 152±22 copies per ng total RNA, respectively (t-test; p=0.009). AVT V1a1 receptor transcript levels were not significantly different between males and females within the POA+H with 29499±3126 and 30019±2943 copies per 100ng total RNA respectively (t-test; p=0.453). Males had significantly higher V1a2 mRNA transcript levels in POA+H than females (61021±4799 and 48705±4043 copies per 100ng total RNA, respectively, t-test; p=0.034). Overall V1a2 transcript levels were 3.6 times higher than V1a1 transcript levels in the POA+H when male and female levels were combined.

Regressions of AVT versus GnRH transcript levels in the POA+H showed significantly different relationships between males and females. Both sexes showed a strong positive relationship between AVT and GnRH with different slopes and R^2 values (Fig. 9; ANCOVA; p=0.038). Regressions of V1a1 versus V1a2 receptor transcript copy number comparing male and female rock hind showed no trend in males and a positive relationship in females (Fig. 10; Regression for females; R^2=0.41, p=0.04).

In males but not females, 11-KT had a strong negative relationship to V1a1 receptor copy number (Regression; R^2=0.922, p=0.009) whereas 11-KT showed a weak positive trend with V1a2. No trends were observed between 11-KT and AVT or GnRH mRNA copy number (Fig. 11).

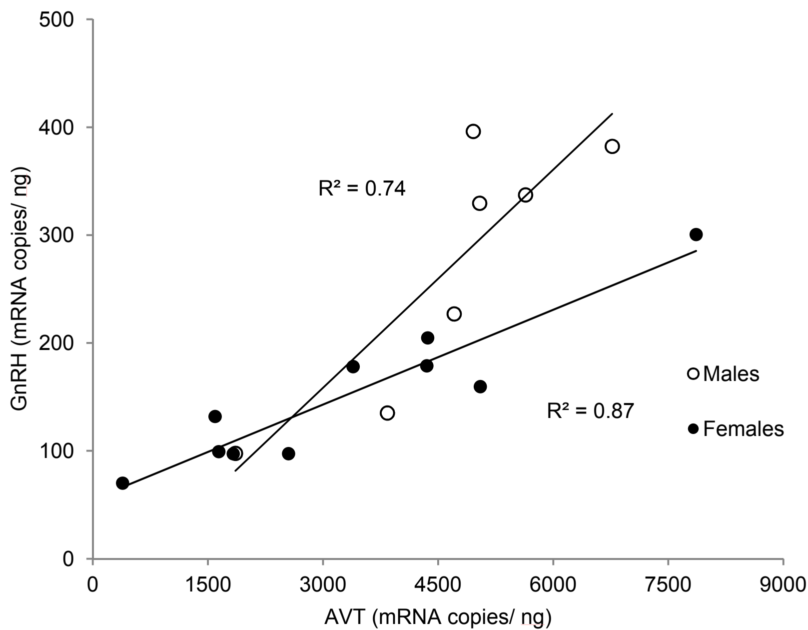

Figure 4.9. Regressions of AVT to GnRH mRNA transcript levels in male and female rock hind. Levels are from the Preoptic area + hypothalamus in copies/ ng total RNA. Regressions for each sex were significantly different from the pooled model (ANCOVA, p= 0.038).

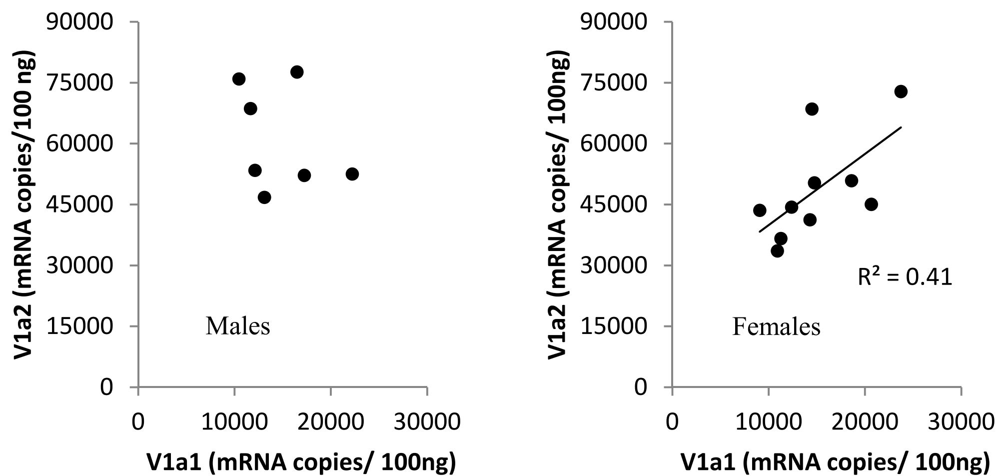

Figure 4.10. Comparison of V1a1 vs. V1a2 receptor transcript levels in male and female rock hind. Levels were measured from the preoptic area+hypothalamus in male and female rock hind demonstrating no relationship in males and a weakly positive relationship in females.

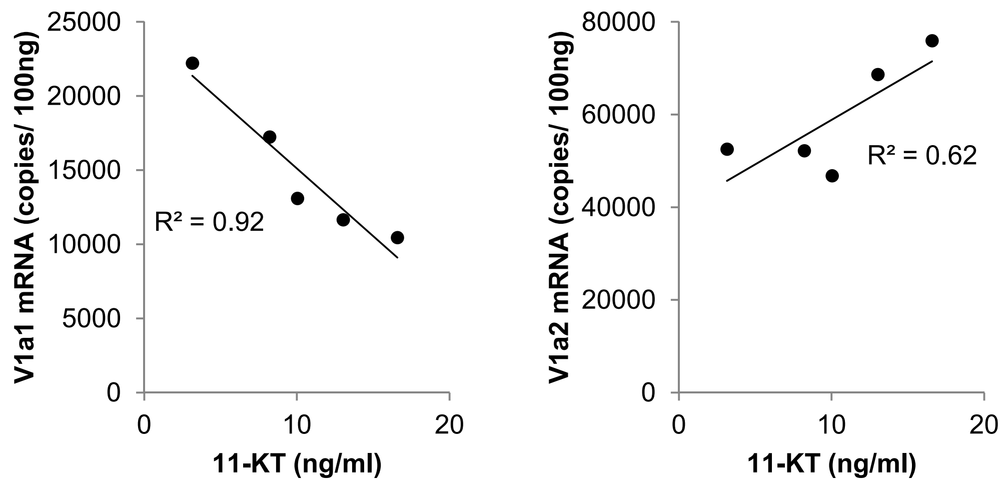

Figure 4.11. Plots of plasma 11-ketotestosterone levels versus mRNA transcript levels for V1a1 and V1a2 receptors. Levels are measured from the preoptic area+hypothalamus in male rock hind demonstrating an inverse relationship.

Early stages of sexual transition of largest female after male removal

In 3d and 10d time series experiments after male removal, sample values for steroid hormones and mRNA transcripts were compared to the initial female condition with ANOVA (Fig. 12). 11-KT values were significantly higher in the 10d large female group (6.05±2.12) than in other groups of female (2.29±0.34), 3d large (2.46±0.89) , 3d small (2.47±0.88) , and 10d small (1.06±0.12 ng per ml plasma) fish (Fig. 10; ANOVA, p= 0.011). Plasma E2 values for the time series compared to initial females were not significantly different. However, unlike the male versus female comparison, 10d large fish that were high in 11-KT also had high levels of E2.

Messenger RNA transcript copy number for AVT, GnRH, V1a1 and V1a2 transcripts did not differ significantly between female, 3d large, 3d small, 10d large, or 10d small groups when compared using ANOVA (Fig. 12). However, the highest

individual values observed for AVT, V1a1 and V1a2 mRNAs occurred in the 10d large group with 677206, 73647 and 80647 copies per ng total RNA, respectively. Rock hind males had the highest recorded values for 11-KT at 15.6 ng per ml and GnRH at 393.2 copies per ng total RNA whereas the highest E2 level was seen in an initial female at 21.4 ng/ml.

In fish sampled at 3d and 10d after male removal, increases in 11-KT levels were seen in some fish. At 3d, 11-KT levels above the maximum in females were observed in two out of five of the largest fish in the groups. At 10d, four out of five of the largest remaining fish had elevated 11-KT levels (Fig. 12). Histological examination of gonadal tissue from these fish revealed that only ovarian tissue was present with little evidence of atresia. However, one fish at the ten day sampling period showed small numbers of spermatocytes were present adjacent to late stage oocytes that were in various stages of degradation.

In the 10 day treatment group, AVT mRNA levels were significantly lower in medium and small fish as compared to the largest remaining fish with 4468±613, 802±276, 2190±550 for large, medium and small fish, respectively (ANOVA on ranks p=0.004 and Dunn's post hoc test: p= 0.005 and p=0.031 for lg vs. med, and lg vs. small comparisons respectively) (Fig. 13). Upon removal for sampling, medium fish had numerous bite marks that were absent in large and small fish.

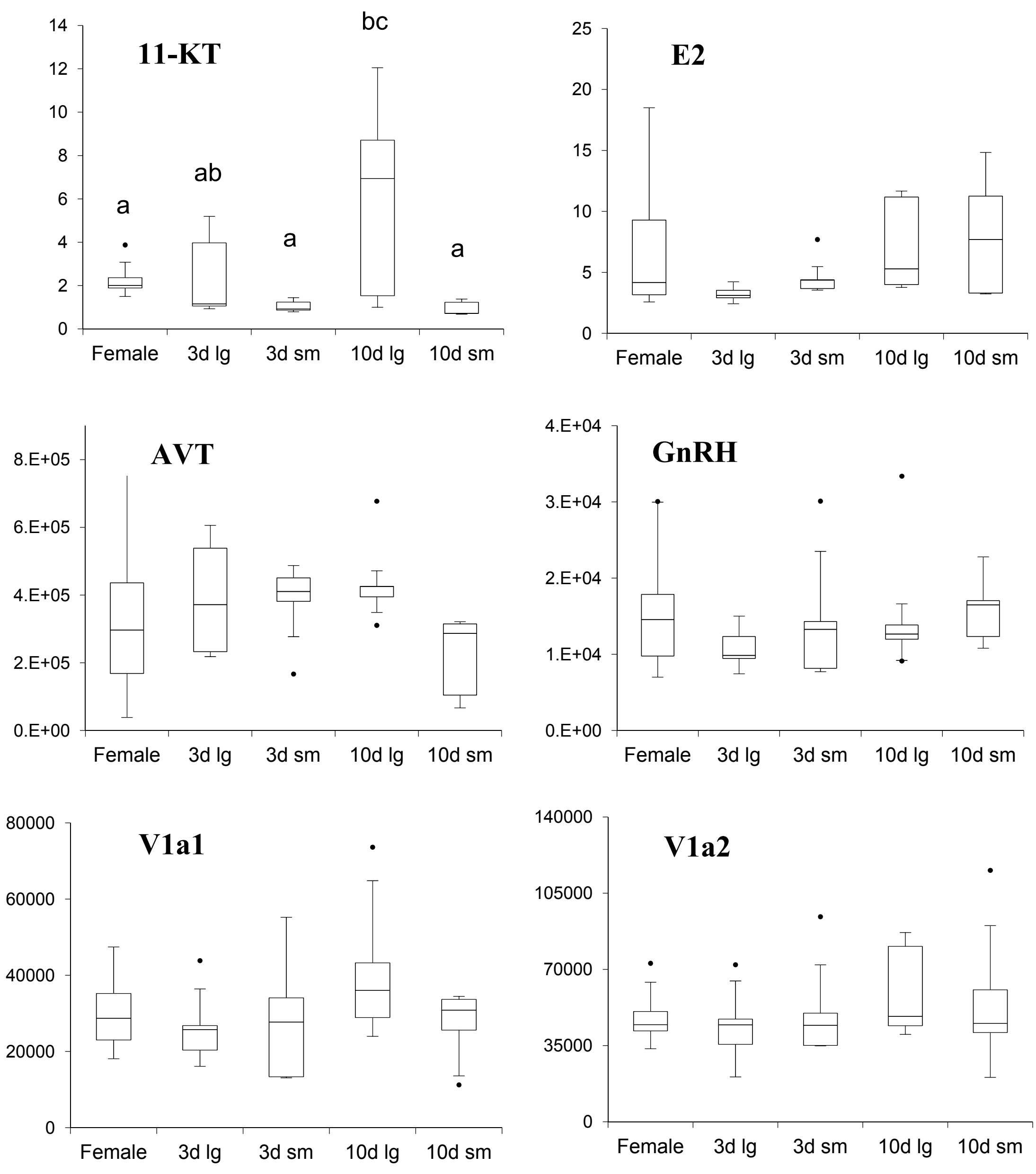

Figure 4.12. Comparison of plasma 11-Ketotestosterone (11-KT), estradiol (E2) (ng/ ml plasma), and mRNA transcript levels for GnRH, AVT, and V1a1 and V1a2 receptors (copies per 100ng total RNA) in the preoptic area+hypothalamus between initial female condition (Female) and the largest and smaller remaining females sampled at 3d and 10d after dominant male removal. The largest fish remaining after male removal is undergoing sex change. Letters indicate significant difference in treatment groups at $p<0.05$ as tested with ANOVA on ranks and Dunn's post-hoc tests. N=8 for Female and N=5 for all other groups.

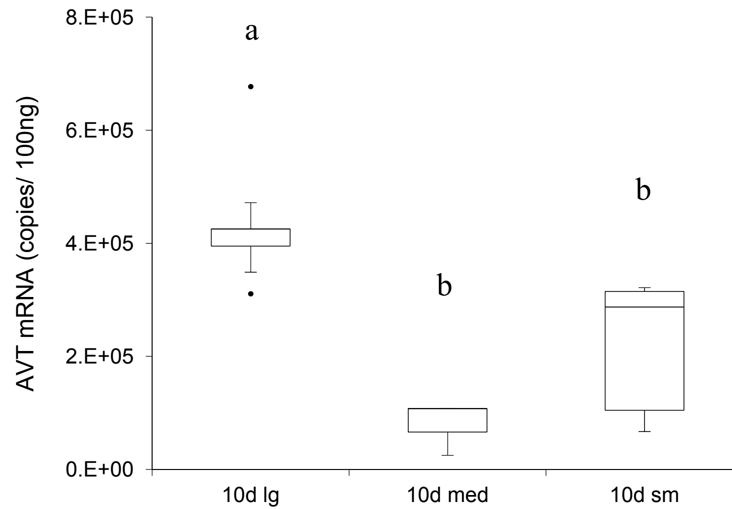

Figure 4.13. Comparison of AVT mRNA transcript levels (copies per 100 ng total RNA) in the preoptic area+hypothalamus between the large medium and small remaining female rock hind sampled at 10d after dominant male removal. Different letters indicate significant differences between groups at $p<0.05$ with ANOVA on ranks and Dunn's post-hoc tests (N= 5, 3, and 5 for lg, med, and small groups respectively).

DISCUSSION

This study provides the first evidence for co-localization of a vasotocin V1a receptor on GnRH-I producing neurons in any vertebrate species. The finding of two distinct V1a receptor types in a perciform species is consistent with similar observation in pupfish (Lema, 2010) and fugu (see table 1). The significantly higher expression of AVT V1a2 receptor and GnRH mRNAs observed in male versus female rock hind together with their co-localization support the possibility of AVT action on GnRH neurons via the V1a2 receptor subtype in this sex changing fish.

The co-localization of the V1a receptor on GnRH neurons in the grouper supports the hypothesis for AVT action on the GnRH system first proposed in chicken by

D'Hondt et al. (2000) based on the proximity of AVT and GnRH producing neurons, but no further research to test the hypothesis has been published since then. Earlier in-vivo studies in rat (Salisbury et al., 1980) and baboon (Koyama and Hagino, 1983) show increases in luteinizing hormone levels due to AVP injection supporting the potential of AVP action on GnRH release. Furthermore, blocking AVP action by injecting V1a receptor antagonists into the third ventricle greatly decreases LH release in the rat (Funabashi et al., 1999), thereby suggesting a possible action of the antagonist on GnRH neurons in close proximity to the injection site. Clearly, further work is needed to confirm effects of AVT on GnRH production and/or release *in vitro* and *in vivo* to establish a functional relationship between the V1a2 receptor and GnRH producing cells in rock hind.

Of the two vasotocin receptors isolated in this study, the rock hind V1a2 sequence shares the most similarity with the functionally characterized V1a receptors from flounder (Warne, 2001) and sucker (Mahlmann et al., 1994b) vasotocin receptors. The highest mRNA expression in rock hind brain for both V1a1 and V1a2 receptors was in the POA+H region and the V1a2 form had higher expression than V1a1 throughout the brain regions tested in this species. The mRNA distribution of V1a2 in all the dissected brain regions was consistent with the IHC localization of this receptor in rock hind brain (Kline, Chapter 3).

The AVT V1a2 but not the V1a1 receptor was detected in the pituitary of rock hind. In contrast, Lema (2010) identified expression of the V1a1 form but not the V1a2 form in the pituitary of pupfish potentially indicating a difference in the function of these receptors. The significant differences in AVT receptor mRNA expression between male and female rock hind in this study for the V1a2 type and not the V1a1 type confirm differential expression between sexes as well. The functional significance of the opposite

trends seen in the two receptor sub-types in relation to circulating 11-KT levels in males is unclear at present. A positive relationship between V1a receptors and androgens has been noted in Syrian hamsters (Young et al., 2000). However, only one form of V1a receptor is currently known in mammals and there are no other data regarding steroid hormones and V1a receptors in fishes.

The results of the male versus female comparison reveal that GnRH and the V1a2 receptor expression on the POA+H may be important components in rock hind male phenotype. This evidence in addition to the co-localization detailed above lends support to the hypothesis of AVT action on GnRH via the V1a2 receptor. Males and Females did not differ in expression of AVT or the V1a1 receptor. Several studies have detailed the variable expression of AVT in different neuron populations however the very high expression of AVT seen in this study in both males and females could mask any small differences in specific cell populations. The sex differences between males and females in V1a2 but not V1a1 expression may indicate a functional difference for the receptors and was also noted in pupfish by Lema (2010).

The lack of significant changes in any of the mRNA transcripts in the time series experiments as compared to the initial female condition was unexpected. It was hypothesized that early in the sex-change process, changes in mRNA levels would be detected during a reorganization of the POA+H from female to male sexual phenotype. Except for changes in the androgen 11-KT in 10d fish, this was not the case. This result could be due to the small numbers of samples in each treatment group or that the hormonal systems have not been sufficiently re-organized to male phenotype. However, it is worth noting that the highest mRNA expressions of AVT, V1a1 and V1a2 receptors, and GnRH were in 10d large fish suggesting there is a trend of increasing expression in this group. Additional approaches such as a longer time period before sampling and

measurement of protein/peptide levels with ELISA in the POAH (Khan et al., 2001) may need to be applied in order to better understand the neuroendocrine mechanisms of sexual transition in this species.

Although multiple forms of the AVT hormone have been identified in the sucker by Morley et al. (1990), no second form has been identified in Perciform fishes to date. A gene duplication in AVT might be an isolated occurrence in Cyprinids and Salmonids and a second form may not exist in rock hind. Nevertheless, every effort was made in this study to avoid homologous regions identified between the cDNA sequences for AVT forms identified in sucker.

The GnRH sequence examined in this study for rock hind was most similar to GnRH-I type sequences it was aligned with. Although this study did not establish the gonadotropin releasing form of GnRH-I in rock hind, the distribution of mRNA transcripts in the brain dissection show that the majority of the GnRH-I transcripts are in the POA+H. Furthermore, sbGnRH-type is the releasing type in other known closely related species tested (Gothilf et al., 1996; Mohamed et al., 2005). The majority of the expression was seen in the POA+H dissection indicating that it is likely the releasing form. However, lower levels of GnRH mRNA in the inferior lobes, pituitary, corpus cerebellum and medulla oblongata may represent expression in GnRH fibers in these tissues in the absence GnRH cell bodies. Similar patterns of GnRH expression in these brain and pituitary tissues have been reported in other Perciform species as discussed in Senthilkumaran et al. (1999a).

Changes in AVT expression have been noted in the bluehead wrasse and cichlid due to sexual phenotype and social status (Godwin et al., 2000; Greenwood et al., 2008). However, significant differences were not detected in AVT mRNA levels in the male versus female or the male removal experiments in the POA+H in rock hind. Since AVT

expression occurs in discrete cell populations that likely serve different functions, a difference in expression in one population would have to overwhelm the expression of other areas to detect a difference in mRNA expression alone. In in-situ hybridization studies differences have been noted in some cell populations and not others within the POAH (Godwin et al., 2000).

One study in cichlid reported conflicting results when examining whole brain versus brain region mRNA expression and this is likely due to these highly variable brain region neuron populations (Greenwood et al., 2008). For example in chicken, a male specific population of AVT producing neurons occurs in the parvocellular region that is not present in females (Jurkevich et al., 1996). Currently there is no information on specific locations of AVT producing cell populations in any grouper species and this warrants further study.

The relationship between AVT and GnRH mRNAs as tested with regression was significantly different for male versus female rock hind, although the two transcripts showed positive correlations in both sexes. These findings suggest the role of GnRH may vary in rock hind based on the sex of the fish. In a separate experiment, GnRH implants in female rock hind shortened the time to display of the male specific “tuxedo” pattern and patrolling behavior to 4 and 6 days after induced spawning and male removal as compared to the typical 32d required (R. Kline unpublished data). This is consistent with similar findings in bluehead wrasse (Kramer et al., 1993) and another grouper, *Epinephelus fario* (Kuo et al., 1988) where injection of GnRH causes precocious sex change.

The significant decreases in AVT expression in medium sized fish and to a lesser extent in small fish at 10d lend support to a role for AVT in male behavior of rock hind. Rock hind males and females are highly aggressive and this may be explained by the

need to control the harem and territory after a dominant male is removed. Similarly sized fish and in some cases intermediate sized females are frequently attacked by the largest remaining female and in extreme cases, can die from their injuries. In 10d fish, bite marks were seen in all medium sized fish from this study.

The reduced levels of AVT expression in smaller fish at 10d do not follow the pattern expected if AVT were associated with the stress axis alone, which should increase (Balment et al., 2006a; Kulczykowska, 2001). However, AVT expression in sex changing fish appears to be associated with sexual phenotype where dominant males have the highest expression of AVT as seen in bluehead wrasse in some cell populations and do not differ in others. AVT levels were not significantly different in the male versus female comparison in this study. However, AVT has also been shown to have a strong relationship to reproductive state, particularly in females (Maruska et al., 2007b) and some female rock hind had the highest levels of AVT mRNA expression measured in this study.

Plasma 11-KT and E2 levels reported here in rock hind are comparable to those reported for males and females of other grouper species (Bhandari et al., 2003). In a study of the honeycomb grouper, Bhandari et al. (2003) reported that 11-KT was the major androgen in that grouper species similar to the situation in rock hind. The increases observed in 11-KT in 10d dominant fish undergoing sexual transition even while still having ovarian tissue with little evidence of atresia is intriguing. These 10d large fish were still phenotypically female because of their high levels of E2 and presence of ovarian tissue. Proposed production sites for 11-KT vary in the literature, e.g., somatic tissue in the gonad, spleen and blood cells, etc. (Alam et al., 2005; Cavaco et al., 1997; Cuisset et al., 1995) , therefore one or more of these other tissues may contribute to the increased 11-KT levels in this group. It was clear at 10d after male removal that fish in

this study still had high levels of estrogen. As seen in wrasse (Higa et al., 2003) and grouper (Bhandari et al., 2005), these E2 levels likely prevented the full transition from female to male and thus far, it is unclear what mechanism is involved in later down-regulation of aromatase expression in rock hind.

Another explanation for the lack of significant differences in mRNA expression of the 10d fish versus initial female or other small fish is that the reproductive state of rock hinds in social groups is highly variable. Even at constant photoperiod and temperature some females would become reproductive after male removal and the rise in E2 seen at 10d may be evidence that aggression plays a role in reproductive cycle in rock hind. As seen in the behavioral study, rock hind are very aggressive to smaller individuals in general, and this increases after male removal and during the spawning period (Kline, chapter 2).

CONCLUSIONS

In summary, co-localization with IHC revealed for the first time that GnRH neurons in the preoptic area express V1a2 receptors, implicating a pathway for AVT to act on GnRH. This finding is a key component for our understanding socially controlled sex change in hermaphroditic fish. Higher levels of the AVT V1a2 receptor and GnRH transcripts in male rock hind also lend evidence to the linkage of these two systems and potentially, a sex specific role for GnRH. In male rock hind, a negative relationship was seen between the V1a1 receptor and plasma 11-KT levels while a positive trend was seen between the V1a2 receptor and 11-KT, indicating that these receptor forms differ in their regulation in males. The presence of elevated levels of 11-KT as well as E2 in the largest remaining females at 10d, in addition to the lack of significant changes seen in any transcript from female levels, indicate that the changes in expression to produce male

phenotype may take longer than the sampling duration used here. Extending the time point for sampling past 10d might also provide more information. In future behavioral experiments with rock hind, a comparison of mRNA transcript levels based on steroid hormone profiles during the sex change process might improve our understanding of this complex process.

Chapter 5: Summary and Conclusions

This study provides important information on the timing and coloration patterns during sexual change in the rock hind that may now be tested in other grouper species. Since many grouper species are targeted worldwide as food fish and removal of males from the population is a serious problem for several species (Coleman et al., 1996), this research may shed light on the time period required for other grouper species to change sex from female to male when the dominant male is removed. If this model of relatively slow transition holds true for larger species, those fish may be able to change sex and spawn when the male removal happens early in the reproductive season but might be more problematic later. This finding is likely to be relevant for effective fisheries management of this important group of fishes.

The finding of two vasotocin V1a receptors in the rock hind brain provides evidence for the first time, that these two forms found in fish from two other orders: Cyprinodontidae (pupfish) and Tetraodontidae (puffer fish) are not isolated incidents (Lema, 2010) and are present in Perciform fish as well. Although the distribution of a V1a receptor has been detailed in the brain of mammals, similar information was lacking for any fish species. The development and testing of a specific antibody for a vasotocin V1a2 receptor in rock hind in this study allowed the finding of extensive expression of the V1a2 receptor in the brain of rock hind and showed that this receptor likely plays a role in numerous neural and integrative functions. This antibody development also allowed the co-localization of the V1a receptor on the surface of GnRH neurons, which provides morphological basis for possible AVT actions on GnRH via this receptor.

The functions of the two distinct V1a receptor forms identified in this study are likely different because of the differential expression seen in relation to androgen levels

in males and the differences seen in male vs. female rock hind. Furthermore, the changes in expression of these two receptors at different time points seen at 10d in large rock hind undergoing sex change and pupfish undergoing osmotic challenge (Lema, 2010) suggests that these receptor forms serve different functions. Future research should include co-localization of these two forms with the development of a V1a1-specific antibody or *in situ* hybridization assays for both forms of these receptors to distinguish cellular distribution in the brain.

The cloning and partial characterization of cDNA sequences for AVT, two forms of the V1a receptor, and GnRH and the development of calibrated assays for each allowed the measurement and comparison of expression between males and females as well as fish in the early stages of sexual change. The lack of significant difference in AVT expression in any group demonstrates that the very high expression of this hormone in the brain of rock hind plays numerous functions in physiological, reproductive and physiological processes (Balment et al., 2006a). Significant differences in GnRH and the V1a2 receptor expression between males and females along with trends seen in receptor transcripts in the early stages of sex change lends support to the hypothesis that an interaction exists between the AVT and GnRH systems. The apparent regulation of the V1a receptor forms in relation to androgen in male rock hind show that the mechanisms present in mammals are likely present in fish as well (Young et al., 2000).

The results from this study also provide baseline data for possible coordinated interactions between the neuropeptide hormones AVT and GnRH to facilitate the process of behavioral and gonadal sex change in protogynous hermaphrodites. In addition these results suggest that arginine vasotocin may play a larger role in social hierarchy as evidenced by the reduction in AVT levels found in the preoptic area and hypothalamus of the brains of subordinate female fish as compared to the female undergoing sex change

10 days after removal of the dominant male. Also, GnRH may play a larger role in male brain phenotype due to the higher mRNA expression detected in male fish.

Although the time series data regarding change in mRNA transcripts were inconclusive in regards to the hypothesis, they did provide further insight into the process of sex change in groupers and confirmed what was observed behaviorally and in comparison to other sex changing species that the process might take longer in groupers than that reported in wrasses and gobies (Grober and Sunobe, 1996; Warner and Swearer, 1991b). This may be due in part to the differing life histories of these species. It may be advantageous to change sex more slowly in rock hind due to the presence of larger fish that may move in to fill the vacancy and the high costs of starting the process of sex change when larger fish are present. This study advances our understanding of the relationship between sex-specific behavior and reproductive development as they relate to the AVT and GnRH systems that are conserved in all vertebrates. It will be important to establish a functional relationship between the two systems in sex changing fishes now that a pathway has been identified and supports the model below:

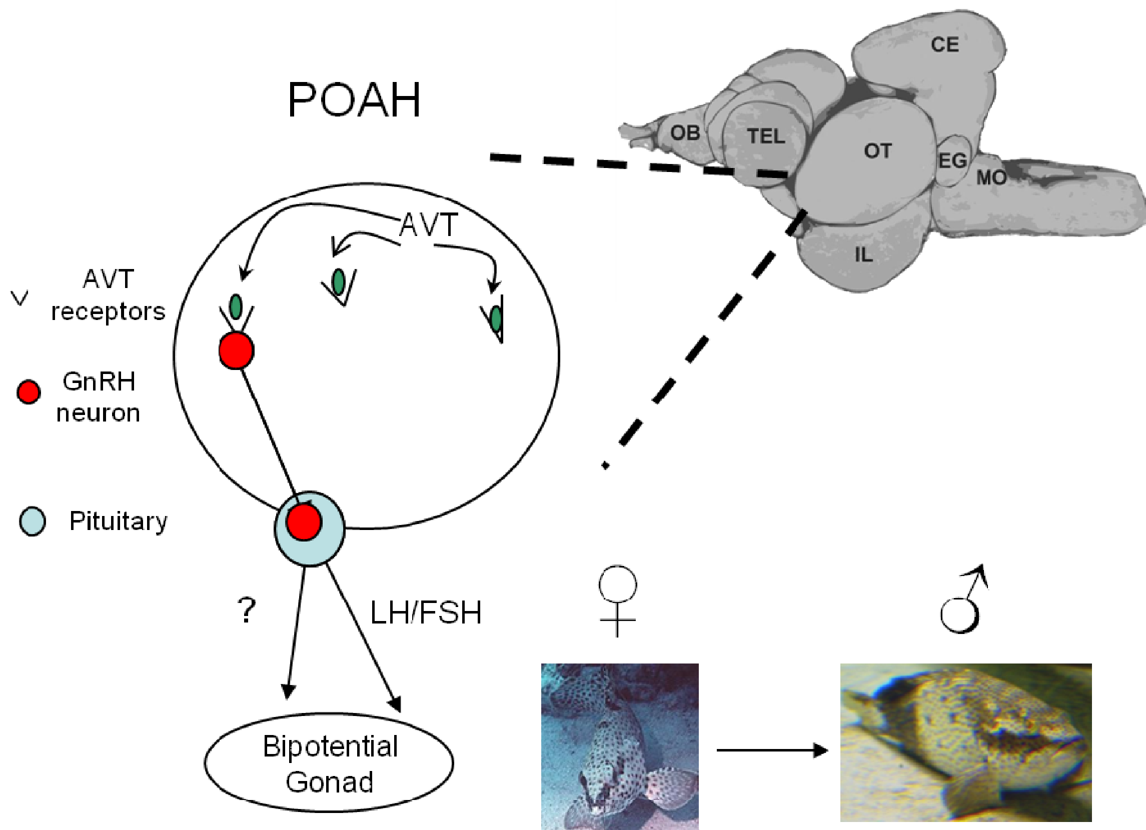

Figure 5.1. A conceptual model for the interaction of the AVT and GnRH systems during rock hind gonadal sex change: When the dominant male is removed from the group, the largest remaining female increases AVT production and release in the preoptic area-anterior hypothalamus (POAH). AVT receptors located on GnRH producing neurons (as shown in this study) cause release of GnRH into the pituitary stimulating the release of gonadotropins (LH/ FSH) and changes in the bipotential gonad, causing sexual change.

Appendices

Appendix A. Rock hind territorial size: two social groups per tank

Territory size and spacing varied from platform to platform, and it is unclear if a minimum territory size or spacing is needed for male rock hind transition. In preliminary studies with rock hind, attempts to establish two social groups were successful by placing two males of similar size in a 3m diameter tank with two PVC structures, two intermediate and six smaller females. Each male rock hind would gradually establish control of a structure and display the tuxedo pattern around a perceived boundary. Periodic encroachment by either male resulted in tuxedo display, mouth fighting and chasing by the other male. Territory development took approximately two weeks when males were placed in a new tank.

Removal of both males from the tank resulted in the largest remaining female establishing control of the entire tank within a few weeks, even when the size difference between the two largest females was small. It appears that competitor size and available habitat play a major role in determining the territorial distribution of this species.

Appendix B. An additional aggression time point

During the development of a new rock hind social group in laboratory experiments, a peak in aggression was observed between 13 and 16 days. Since many of the fish collected for experiments were from different social groups in the wild, a period of social organization was apparently needed for the establishment of dominance. Between 13 and 16 days, the largest female would often show signs of bite marks and

tissue missing from the tail. In several cases, the female was so badly damaged that it died from its injuries and the experiment was restarted with more fish. Gonadal histology on these fish revealed that they were female with ovarian tissue with no evidence of sexual change. This two week time point appears to have similarities between males acquiring new territories and females transitioning to males as the second largest remaining female often showed physical damage in the male removal experiments at the same time period.

References

Alam MA, Komuro H, Bhandari RK, Nakamura S, Soyano K, Nakamura M. 2005. Immunohistochemical evidence identifying the site of androgen production in the ovary of the protogynous grouper *Epinephelus merra*. Cell Tissue Res 320:323-329.

Albers HE, Pollock J, Simmons WH, Ferris CF. 1986. A V1-like receptor mediates vasopressin-induced flank marking behavior in hamster hypothalamus. J Neurosci 6:2085–2089.

Baeyens DA, Cornett LE. 2006. The cloned avian neurohypophysial hormone receptors. Comp Biochem Phys B 143:12–19.

Balment RJ, Lu W, Weybourne E, Warne JM. 2006a. Arginine vasotocin a key hormone in fish physiology and behaviour: A review with insights from mammalian models. Gen Comp Endocr 147:9–16.

Balment RJ, Lu W, Weybourne E, Warne JM. 2006b. Arginine vasotocin a key hormone in fish physiology and behaviour: A review with insights from mammalian models. General and Comparative Endocrinology 147:9-16.

Bass AH, Grober MS. 2001. Social and neural modulation of sexual plasticity in teleost fish. Brain Behavior and Evolution 57:293-300.

Bhandari RK, Alam MA, Higa M, Soyano K, Nakamura M. 2005. Evidence that estrogen regulates the sex change of honeycomb grouper (*Epinephelus merra*), a protogynous hermaphrodite fish. J Exp Zool Part A 303A:497-503.

Bhandari RK, Komuro H, Higa M, Nakamura M. 2004. Sex inversion of sexually immature honeycomb grouper (*Epinephelus merra*) by aromatase inhibitor. Zool Sci 21:305-310.

Bhandari RK, Komuro H, Nakamura S, Higa M, Nakamura M. 2003. Gonadal restructuring and correlative steroid hormone profiles during natural sex change in protogynous honeycomb grouper (*Epinephelus merra*). Zool Sci 20:1399-1404.

Birnbaumer M. 2000. Vasopressin receptors. Trends Endocrin Met 11:406–410.

Black M, Balthazart J, Baillien M, Grober M. 2005. Socially induced and rapid increases in aggression are inversely related to brain aromatase activity in a sex-changing fish, *Lythrypnus dalli*. Proceedings of the Royal Society B: Biological Sciences 272:2435-2440.

Boyd SK. 1991. Effect of vasotocin on locomotor activity in bullfrogs varies with developmental stage and sex. Horm Behav 25:57–69.

Boyd SK. 1997. Brain vasotocin pathways and the control of sexual behaviors in the bullfrog. Brain Res Bull 44:345-350.

Braford MRJ, Northcutt RG. 1983. Organization of the diencephalon and pretectum of the ray-finned fishes. In: Northcutt RG, Davis RE, editors. Fish neurobiology. Ann Arbor: University of Michigan Press. p 117–163.

Bradley BJ, Mundy NI. 2008. The primate palette: The evolution of primate coloration. Evolutionary Anthropology: Issues, News, and Reviews 17:97-111.

Brantley RK, Bass AH. 1994. Alternative male spawning tactics and acoustic signals in the plainfin midshipman fish *Porichthys notatus* girard (Teleostei, Batrachoididae). Ethology 96:213-232.

Burns KJ. 1998. A phylogenetic perspective on the evolution of sexual dichromatism in tanagers (Thraupidae): The role of female versus male plumage. Evolution 52:1219-1224.

Cardwell JR, Liley NR. 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. Gen Comp Endocr 81:7-20.

Carneiro LA, Oliveira RF, Canario AVM, Grober MS. 2003. The effect of arginine vasotocin on courtship behaviour in a blenniid fish with alternative reproductive tactics. Fish Physiology and Biochemistry 28:241-243.

Cavaco J, Lambert J, Schulz R, Goos H. 1997. Pubertal development of male african catfish, *Clarias gariepinus*. *In vitro* steroidogenesis by testis and interrenal tissue and plasma levels of sexual steroids. Fish Physiol Biochem 16:129-138.

Cerda-Reverter JM, Muriach B, Zanuy S, Munoz-Cueto JA. 2008. A cytoarchitectonic study of the brain of a perciform species, the sea bass (*Dicentrarchus labrax*): The midbrain and hindbrain. Acta Histochem 110:433–450.

Cerda-Reverter JM, Zanuy S, Munoz-Cueto JA. 2001a. Cytoarchitectonic study of the brain of a perciform species, the sea bass (*Dicentrarchus labrax*). I. The telencephalon. J Morphol 247:217–228.

Cerda-Reverter JM, Zanuy S, Munoz-Cueto JA. 2001b. Cytoarchitectonic study of the brain of a perciform species, the sea bass (*Dicentrarchus labrax*). Ii. The diencephalon. J Morphol 247:229–251.

Coleman FC, Koenig CC, Collins LA. 1996. Reproductive styles of shallow-water groupers (Pisces:Serranidae) in the eastern gulf of mexico and the consequences of fishing spawning aggregations. Environ Biol Fish 47:129-141.

Colin PL, Shapiro DY, Weiler D. 1987. Aspects of the reproduction of two groupers, *Epinephelus guttatus* and *e. Striatus* in the west indies. B Mar Sci 40:220-230.

Conklin DJ, Smith MP, Olson KR. 1999. Pharmacological characterization of arginine vasotocin vascular smooth muscle receptors in the trout (*Oncorhynchus mykiss*) *in vitro*. Gen Comp Endocr 114:36–46.

Cuisset B, Fostier A, Williot P, Bennetau-Pelissero C, Menn F. 1995. Occurrence and *in vitro* biosynthesis of 11-ketotestosterone in Siberian sturgeon, *Acipenser baeri* brandt maturing females. Fish Physiol Biochem 14:313-322.

D'Hondt E, Eelen M, Berghman L, Vandesande F. 2000. Colocalization of arginine-vasotocin and chicken luteinizing hormone-releasing hormone-I (cLHRH-I) in the preoptic-hypothalamic region of the chicken. Brain Research 856:55-67.

Dawkins MS, Guilford T. 1993. Colour and pattern in relation to sexual and aggressive behaviour in the bluehead wrasse *Thalassoma bifasciatum*. Behavioural Processes 30:245-251.

Demski LS, Dulka JG. 1986. Thalamic stimulation evokes sex-color change and gamete release in a vertebrate hermaphrodite. Experientia 42:1285–1287.

Devlin RH, Nagahama Y. 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. Aquaculture 208:191-364.

Dubois EA, Zandbergen MA, Peute J, Goos HJT. 2002. Evolutionary development of three gonadotropin-releasing hormone (gnrh) systems in vertebrates. Brain Research Bulletin 57:413-418.

Elofsson U, Winberg S, Francis RC. 1997. Number of preoptic gnrh-immunoreactive cells correlates with sexual phase in a protandrously hermaphroditic fish, the dusky anemonefish (*Amphiprion melanopus*). Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology V181:484-492.

Foran CM, Bass AH. 1999a. Preoptic GnRH and AVT: Axes for sexual plasticity in teleost fish. General and Comparative Endocrinology 116:141-152.

Fryer JN, Boudreault-Chateauvert C, Kirby RP. 1985. Pituitary afferents originating in the paraventricular organ (PVO) of the goldfish hypothalamus. J Comp Neurol 242:475-484.

Funabashi T, Aiba S, Sano A, Shinohara K, Kimura F. 1999. Intracerebroventricular injection of arginine-vasopressin v1 receptor antagonist attenuates the surge of luteinizing hormone and prolactin secretion in proestrous rats. Neuroscience Letters 260:37-40.

Gilmore GR, Jones RS. 1992. Color variation and associated behavior in the epinepheline groupers, *Mycteroperca microlepis* (Goode and Bean) and *m. Phenax* (Jordan and Swain). B Mar Sci 51:83-103.

Godwin J. 2009. Social determination of sex in reef fishes. Seminars in Cell & Developmental Biology 20:264-270.

Godwin J, Sawby R, Warner RR, Crews D, Grober MS. 2000. Hypothalamic arginine vasotocin mrna abundance variation across sexes and with sex change in a coral reef fish. Brain Behav Evolut 55:77–84.

Goodson JL, Bass AH. 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. Nature 403:769-772.

Goodson JL, Bass AH. 2001. Social behavior functions and related anatomical characteristics of vasotocin/vasopressin systems in vertebrates, (vol 35, pg 246, 2001). Brain Research Reviews 36:91–94.

Gore AC. 2002. Gnrh, the master molecule of reproduction. Boston: Kluwer Academic Publishers. xx, 324 p. p.

Gothilf Y, MunozCueto JA, Sagrillo CA, Selmanoff M, Chen TT, Kah O, Elizur A, Zohar Y. 1996. Three forms of gonadotropin-releasing hormone in a Perciform fish (*Sparus aurata*): Complementary deoxyribonucleic acid characterization and brain localization. Biol Reprod 55:636–645.

Greenwood AK, Wark AR, Fernald RD, Hofmann HA. 2008. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an african cichlid fish. Proceedings of the Royal Society B: Biological Sciences 275:2393-2402.

Grober MS, George AA, Watkins KK, Carneiro LA, Oliveira RF. 2002. Forebrain AVT and courtship in a fish with male alternative reproductive tactics. Brain Res Bull 57:423–425.

Grober MS, Sunobe T. 1996. Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. Neuroreport 7:2945–2949.

Groves DJ, Batten TF. 1986. Direct control of the gonadotroph in a teleost, *Poecilia latipinna*. Ii. Neurohormones and neurotransmitters. Gen Comp Endocrinol 62:315–326.

Hastings RW, Ogren LH, Mabry MT. 1976. Observations on the fish fauna associated with offshore platforms in the northeastern gulf of mexico. Fish Bull; Journal 74:2:387-402.

Hasunuma I, Sakai T, Nakada T, Toyoda F, Namiki H, Kikuyama S. 2007. Molecular cloning of three types of arginine vasotocin receptor in the newt, *Cynops pyrrhogaster*. Gen Comp Endocr 151:252–258.

Hayashi M, Sasaki S, Tsuganezawa H, Monkawa T, Kitajima W, Konishi K, Fushimi K, Marumo F, Saruta T. 1994. Expression and distribution of aquaporin of collecting duct are regulated by vasopressin V-2 receptor in rat-kidney. J Clin Invest 94:1778-1783.

Higa M, Ogasawara K, Sakaguchi A, Nagahama Y, Nakamura M. 2003. Role of steriod hormones in sex change of protogynous wrasse. Fish Physiol Biochem 28:149-150.

Insel TR, Wang ZX, Ferris CF. 1994. Patterns of brain vasopressin receptor distribution associated with social-organization in microtine rodents. J Neurosci 14:5381–5392.

Iwata E, Nagai Y, Sasaki H. 2008. Social rank modulates brain arginine vasotocin immunoreactivity in false clown anemonefish (*Amphiprion ocellaris*). Fish Physiol Biochem 36: 337-345.

Jones DT. 2007. Improving the accuracy of transmembrane protein topology prediction using evolutionary information. Bioinformatics 23:538-544.

Jurkevich A, Barth SW, Grossmann R. 1996. Sexual dimorphism of arg-vasotocin gene expressing neurons in the telencephalon and dorsal diencephalon of the domestic fowl. An immunocytochemical and in situ hybridization study. Cell Tissue Res 287:69-77.

Jurkevich A, Berghman LR, Cornett LE, Kuenzel WJ. 2005. Characterization and immunohistochemical visualization of the vasotocin VT2 receptor in the pituitary gland of the chicken, *Gallus gallus*. Gen Comp Endocr 143:82–91.

Kah O, Chambolle P. 1983. Serotonin in the brain of the goldfish, *Carassius auratus*. An immunocytochemical study. Cell Tissue Res 234:319–333.

Khan IA, Mathews S, Okuzawa K, Kagawa H, Thomas P. 2001. Alterations in the GnRH-LH system in relation to gonadal stage and aroclor 1254 exposure in Atlantic croaker. Comp Biochem Physiol B Biochem Mol Biol 129:251-259.

Khan IA, Thomas P. 1993. Immunocytochemical localization of serotonin and gonadotropin-releasing-hormone in the brain and pituitary-gland of the atlantic croaker *Micropogonias undulatus*. Gen Comp Endocr 91:167–180.

Kline RJ, Khan IA, Soyano K, Takushima M. 2008. Role of follicle-stimulating hormone and androgens on the sexual inversion of sevenband grouper *Epinephelus septemfasciatus*. North American Journal of Aquaculture 70:266-272.

Koyama T, Hagino N. 1983. The effect of vasopressin on LH release in baboons. Horm Metab Res 15:184-186.

Kramer CR, Caddell MT, Bubenheimerlivolsi L. 1993. sGnRH-a[(d-arg6,pro9,net-)LHRH] in combination with domperidone induces gonad reversal in a protogynous fish, the bluehead wrasse, *Thalassoma bifasciatum*. J Fish Biol 42:185-195.

Kroeber S, Schomerus C, Korf HW. 1998. A specific and sensitive double-immunofluorescence method for the demonstration of s-antigen and serotonin in trout and rat pinealocytes by means of primary antibodies from the same donor species. Histochemistry and Cell Biology 109:309-317.

Kulczykowska E. 1995. Arginine vasotocin-melatonin interactions in fish: A hypothesis. Rev Fish Biol Fish 5:96–102.

Kulczykowska E. 2001. Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (*Oncorhynchus mykiss*). Fish Physiol Biochem 24:201-206.

Kulczykowska E, Warne JM, Balment RJ. 2001. Day-night variations in plasma melatonin and arginine vasotocin concentrations in chronically cannulated flounder (*Platichthys flesus*). Comp Biochem Physiol A Mol Integr Physiol 130:827-834.

Kuo CM, Ting YY, Yeh SL. 1988. Induced sex reversal and spawning of blue-spotted grouper, *Epinephelus fario*. Aquaculture 74:113-126.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.

Lema SC. 2010. Identification of multiple vasotocin receptor cdnas in teleost fish: Sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge. Mol Cell Endocrinol 321:215–230.

Lema SC, Nevitt GA. 2004. Exogenous vasotocin alters aggression during agonistic exchanges in male amargosa river pupfish (*Cyprinodon nevadensis amargosae*). Horm Behav 46:628–637.

Lethimonier C, Madigou T, Muñoz-Cueto J-A, Lareyre J-J, Kah O. 2004. Evolutionary aspects of GnRHs, GnRH neuronal systems and gnrh receptors in Teleost fish. Gen Comp Endocr 135:1-16.

Lewis CM, Dolence EK, Hubbard CS, Rose JD. 2005. Identification of roughskin newt medullary vasotocin target neurons with a fluorescent vasotocin conjugate. J Comp Neurol 491:381–389.

Liley NR, Stacey NE. 1983. Hormones, pheromones, and reproductive-behavior in fish. Fish Physiol 9:1-63.

Liu M, Sadovy Y. 2004a. Early gonadal development and primary males in the protogynous Epinepheline, *Cephalopholis boenak*. Journal of Fish Biology 65:987-1002.

Liu M, Sadovy Y. 2004b. The influence of social factors on adult sex change and juvenile sexual differentiation in a diandric, protogynous Epinepheline, *Cephalopholis boenak* (pisces, serranidae). J Zool 264:239-248.

Liu YC, Salamone JD, Sachs BD. 1997. Lesions in medial preoptic area and bed nucleus of stria terminalis: Differential effects on copulatory behavior and noncontact erection in male rats. J Neurosci 17:5245–5253.

Lntnesky MMF. 1994. Density-dependent protogynous sex change in territorial-haremic fishes: Models and evidence. Behavioral Ecology 5:375-383.

Lutnesky M. 1988. Sexual dimorphism, dichromatism, and protogynous hermaphroditism in the pomacanthid angelfish, *Centropyge potteri*. Pacific Science 42:46-48.

Maan ME, Eshuis B, Haesler MP, Schneider MV, van Alphen JJM, Seehausen O. 2008. Color polymorphism and predation in a lake victoria cichlid fish. Copeia:621-629.

Mackie MC. 2003. Socially controlled sex-change in the half-moon grouper, *Epinephelus rivulatus*, at ningaloo reef, western australia. Coral Reefs 22:133-142.

Mahlmann S, Meyerhof W, Hausmann H, Heierhorst J, Schonrock C, Zwiers H, Lederis K, Richter D. 1994a. Structure, function, and phylogeny of [arg(8)]vasotocin receptors from teleost fish and toad. P Natl Acad Sci USA 91:1342-1345.

Maney DL, Goode CT, Wingfield JC. 1997. Intraventricular infusion of arginine vasotocin induces singing in a female songbird. J Neuroendocrinol 9:487-491.

Manning M, Sawyer WH. 1993. Design, synthesis and some uses of receptor-specific agonists and antagonists of vasopressin and oxytocin. J Receptor Res 13:195–214.

Marler CA, Boyd SK, Wilczynski W. 1999. Forebrain arginine vasotocin correlates of alternative mating strategies in cricket frogs. Horm Behav 36:53-61.

Maruska KP, Mizobe MH, Tricas TC. 2007a. Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the brain of the halfspotted goby. Comp Biochem Phys A 147:129-144.

Miller BH, Olson SL, Levine JE, Turek FW, Horton TH, Takahashi JS. 2006. Vasopressin regulation of the proestrous luteinizing hormone surge in wild-type and clock mutant mice. Biol Reprod 75:778-784.

Miranda LA, Montaner AD, Ansaldo M, Affanni JM, Somoza GM. 1999. Characterization of brain gonadotropin-releasing hormone (GnRH) molecular variants in brain extracts from different perciform fishes from antarctic waters. Polar Biol 21:122–127.

Mohamed JS, Thomas P, Khan IA. 2005. Isolation, cloning, and expression of three prepro-GnRH mRNAs in Atlantic croaker brain and pituitary. J Comp Neurol 488:384–395.

Moons L, Cambre M, Batten TFC, Vandesande F. 1989. Autoradiographic localization of binding-sites for vasotocin in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Neurosci Lett 100:11–16.

Moore FL, Miller LJ. 1983. Arginine vasotocin induces sexual-behavior of newts by acting on cells in the brain. Peptides 4:97–102.

Morley SD, Schonrock C, Heierhorst J, Figueroa J, Lederis K, Richter D. 1990. Vasotocin genes of the teleost fish catostomus commersoni: Gene structure, exon-intron boundary, and hormone precursor organization. Biochemistry 29:2506-2525.

Munoz RC, Warner RR. 2003. A new version of the size-advantage hypothesis for sex change: Incorporating sperm competition and size-fecundity skew. American Naturalist 161:749-761.

Muske LE, Fernald RD. 1987a. Control of a teleost social signal .1. Neural basis for differential expression of a color pattern. J Comp Physiol A 160:89-97.

Muske LE, Fernald RD. 1987b. Control of a teleost social signal .2. Anatomical and physiological specializations of chromatophores. J Comp Physiol A 160:99-107.

Nair HP, Young LJ. 2006. Vasopressin and pair-bond formation: Genes to brain to behavior. Physiology (Bethesda) 21:146–152.

Nakamura M, Alam MA, Kobayashi Y, Bhandari RK. 2007. Role of sex hormones in sex change of grouper. J Mar Sci Technol-Ta 15:23-27.

Nakamura M, Bhandari RK, Higa M. 2003. The role estrogens play in sex differentiation and sex changes of fish. Fish Physiology and Biochemistry 28:113-117.

Neidig CL, Skapura DP, Grier HJ, Dennis CW. 2000. Techniques for spawning common snook: Broodstock handling, oocyte staging, and egg quality. North American Journal of Aquaculture 62:103-113.

Noga EJ. 2000. Fish disease : Diagnosis and treatment. Ames: Iowa State University. ix, 367 p. p.

Northcutt RG, Davis RE. 1983. Telencephalic organization in ray-finned fishes. In: Davis RE, Northcutt RG, editors. Fish neurobiology. Ann Arbor: University of Michigan Press. p 203–236.

Ogawa S, Akiyama G, Kato S, Soga T, Sakuma Y, Parhar IS. 2006. Immunoneutralization of gonadotropin-releasing hormone type-III suppresses male reproductive behavior of cichlids. Neuroscience Letters 403:201-205.

Orcel H, Tobin VA, Alonso G, Rabie A. 2002. Immunocytochemical localization of vasopressin V1a receptors in the rat pituitary gonadotropes. Endocrinology 143:4385-4388.

Ostrowski NL, Lolait SJ, Young WS. 1994. Cellular-localization of vasopressin V1a receptor messenger-ribonucleic-acid in adult male-rat brain, pineal, and brain vasculature. Endocrinology 135:1511–1528.

Parhar IS, Tosaki H, Sakuma Y, Kobayashi M. 2001. Sex differences in the brain of goldfish: Gonadotropin-releasing hormone and vasotocinergic neurons. Neuroscience 104:1099-1110.

Polovina JJ, Ralston S. 1987. Tropical snappers and groupers : Biology and fisheries management. Boulder: Westview Press. x, 659 p. p.

Potts JC, Manooch CS. 1995. Age and growth of red hind and rock hind collected from north-carolina through the dry-tortugas, florida. B Mar Sci 56:784-794.

Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM. 1994. 3 forms of gonadotropin-releasing-hormone characterized from brains of one species. P Natl Acad Sci USA 91:12081-12085.

Propper CR, Dixon TB. 1997. Differential effects of arginine vasotocin and gonadotropin-releasing hormone on sexual behaviors in an anuran amphibian. Horm Behav 32:99–104.

Rink E, Wullimann MF. 2002. Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (*Danio rerio*) lead to identification of an ascending dopaminergic system in a teleost. Brain Res Bull 57:385-387.

Robertson DR. 1972. Social control of sex reversal in a coral-reef fish. Science 177:1007-1009.

Rooker JR, Dokken QR, Pattengill CV, Holt GJ. 1997. Fish assemblages on artificial and natural reefs in the flower garden banks national marine sanctuary, USA. Coral Reefs 16:83-92.

Ross RM, Losey GS, Diamond M. 1983. Sex change in a coral-reef fish - dependence of stimulation and inhibition on relative size. Science 221:574-575.

Sadovy Y, Colin PL, Domeier ML. 1994. Aggregation and spawning in the tiger grouper, *Mycteroperca tigris* (Pisces, Serranidae). Copeia:511-516.

Sadovy Y, Shapiro DY. 1987. Criteria for the diagnosis of hermaphroditism in fishes. Copeia:136-156.

Saito D, Hasegawa Y, Urano A. 2003. Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout. Neuroscience Letters 351:107-110.

Sakai Y, Karino K, Kuwamura T, Nakashima Y, Maruo Y. 2003. Sexually dichromatic protogynous angelfish *Centropyge ferrugata* (Pomacanthidae) males can change back to females. Zool Sci 20:627-633.

Sakai Y, Kuniyoshi H, Yoshida M, Fukui Y, Hashimoto H, Gushima K. 2007. Social control of terminal phase transition in primary males of the diandric wrasse, *Halichoeres poecilopterus* (Pisces: Labridae). J Ethol 25:57-61.

Salek SJ, Sullivan CV, Godwin J. 2002. Arginine vasotocin effects on courtship behavior in male white perch (*Morone americana*). Behav Brain Res 133:177–183.

Salisbury RL, Krieg RJ, Jr., Seibel HR. 1980. Effects of arginine vasotocin, oxytocin, and arginine vasopressin on steroid-induced surges of luteinizing hormone and prolactin in ovariectomized rats. Acta Endocrinol 94:166-173.

Santangelo N, Bass AH. 2006. New insights into neuropeptide modulation of aggression: Field studies of arginine vasotocin in a territorial tropical damselfish. P R Soc B 273:3085–3092.

Semsar K, Godwin J. 2003. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. J Neurosci 23:4386-4393.

Semsar K, Godwin J. 2004a. Multiple mechanisms of phenotype development in the bluehead wrasse. Horm Behav 45:345-353.

Semsar K, Kandel FLM, Godwin J. 2001a. Manipulations of the avt system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Horm Behav 40:21–31.

Senthilkumaran, Okuzawa, Gen, Ookura, Kagawa. 1999a. Distribution and seasonal variations in levels of three native gnrhs in the brain and pituitary of perciform fish. J Neuroendocrinol 11:181-186.

Serradeil-Le Gal C, Wagnon J, Valette G, Garcia G, Pascal M, Maffrand JP, Le Fur G. 2002. Nonpeptide vasopressin receptor antagonists: Development of selective and orally active v1a, v2 and v1b receptor ligands. Prog Brain Res 139:197–210.

Shapiro DY. 1981a. Sequence of coloration changes during sex reversal in the tropical marine fish *Anthias squamipinnis* (Peters). B Mar Sci 31:383-398.

Shapiro DY. 1981b. Size, maturation and the social-control of sex reversal in the coral-reef fish *Anthias squamipinnis*. J Zool 193:105-128.

Siebeck UE. 2004. Communication in coral reef fish: The role of ultraviolet colour patterns in damselfish territorial behaviour. Anim Behav 68:273-282.

Stehle J, Reuss S, Riemann R, Seidel A, Vollrath L. 1991. The role of arginine-vasopressin for pineal melatonin synthesis in the rat: Involvement of vasopressinergic receptors. Neurosci Lett 123:131-134.

Strader CD, Fong TM, Tota MR, Underwood D, Dixon RAF. 1994. Structure and function of g-protein-coupled receptors. Annual Review of Biochemistry 63:101-132.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599.

Tanoue A, Ito S, Honda K, Oshikawa S, Kitagawa Y, Koshimizu T, Mori T, Tsujimoto G. 2004. The vasopressin v1b receptor critically regulates hypothalamic-pituitary-adrenal aids activity under both stress and resting conditions. J Clin Invest 113:302–309.

Thompson RR, Moore FL. 2000. Vasotocin stimulates appetitive responses to the visual and pheromonal stimuli used by male roughskin newts during courtship. Horm Behav 38:75–85.

Thresher RE. 1984. Reproduction in reef fishes. Neptune City, NJ: T.F.H. Publications. 399 p.

Todd BD, Davis AK. 2007. Sexual dichromatism in the marbled salamander *Ambyastoma opacum*. Can J Zool 85:1008-1013.

Vargas JP, Bingman VP, Portavella M, Lopez JC. 2006. Telencephalon and geometric space in goldfish. Eur J Neurosci 24:2870–2878.

Walton JC, Waxman B, Hoffbuhr K, Kennedy M, Beth E, Scangos J, Thompson RR. 2010. Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. Neuropharmacology 58:126–134.

Warne JM. 2001. Cloning and characterization of an arginine vasotocin receptor from the euryhaline flounder *Platichthys flesus*. Gen Comp Endocr 122:312–319.

Warne JM, Bond H, Weybourne E, Sahajpal V, Lu W, Balment RJ. 2005. Altered plasma and pituitary arginine vasotocin and hypothalamic provasotocin expression in flounder (*Platichthys flesus*) following hypertonic challenge and distribution of vasotocin receptors within the kidney. Gen Comp Endocr 144:240–247.

Warner RR. 1982. Mating systems, sex change and sexual demography in the rainbow wrasse, *Thalassoma lucasanum*. Copeia 1982:653-661.

Warner RR. 1984. Mating-behavior and hermaphroditism in coral-reef fishes. Am Sci 72:128-136.

Warner RR, Schultz ET. 1992. Sexual selection and male characteristics in the bluehead wrasse, *Thalassoma bifasciatum* - mating site acquisition, mating site defense, and female choice. Evolution 46:1421-1442.

Warner RR, Swearer SE. 1991a. Social-control of sex-change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces, Labridae). Biological Bulletin 181:199-204.

White SA, Nguyen T, Fernald RD. 2002. Social regulation of gonadotropin-releasing hormone. Journal of Experimental Biology 205:2567-2581.

Winslow JT, Hastings N, Carter CS, Harbaugh CR, Insel TR. 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. Nature 365:545-548.

Wullimann MF. 1998. The central nervous system. In: Evans DH, editor. The physiology of fishes. 2nd ed. Boca Raton, Florida,: CRC Press. p 247–284.

Wullimann MF, Rupp B, Reichert H. 1996. Neuroanatomy of the zebrafish brain : A topological atlas. Basel ; Boston: Birkhäuser Verlag. vi, 144 p. p.

Yamamoto N, Oka Y, Kawashima S. 1997. Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells: Effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinology 65:403–412.

Yeh S-L, Kuo C-M, Ting Y-Y, Chang C-F. 2003. The effects of exogenous androgens on ovarian development and sex change in female orange-spotted protogynous grouper, *Epinephelus coioides*. Aquaculture 218:729-739.

Young LJ, Wang Z, Cooper TT, Albers HE. 2000. Vasopressin (V1a) receptor binding, mRNA expression and transcriptional regulation by androgen in the syrian hamster brain. J Neuroendocrinol 12:1179-1185.

Yu KL, He ML, Chik CC, Lin XW, Chang JP, Peter RE. 1998. mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. General and Comparative Endocrinology 112:303-311.

Vita

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